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Localization and trafficking of Pannexin 1 in polarized and non-polarized cells

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Abstract

The channel-forming membrane protein pannexin 1 (Panx1) is best characterized as an ATP release channel and has been linked to over a dozen human pathologies. Along with protein-protein interactions and post-translational modifications, the physiological function of Panx1 channels is highly dictated by its subcellular localization. In polarized epithelial cells *in vivo*, Panx1 is reported to selectively localize to distinct plasma membrane domains. Here, we investigated whether this polarized distribution is guided by internal motifs contained within the Panx1 polypeptide. In polarized MDCK cells, Panx1 was localized predominantly at the apical membrane domain, although some remained detectable in the basolateral cell surface. In non-polarized cells, Panx1 localized throughout the entire plasma membrane, including the lamellipodia of migratory tumor cells. The membrane distribution of Panx1 remained unchanged upon mutation of a dileucine motif in the C-terminal domain, indicating that this motif is not responsible for basolateral Panx1 protein sorting in polarized cells. Interestingly, stable expression of a Panx1 mutant where a putative tyrosine-based sorting motif was eliminated caused MDCK cells to lose the ability to polarize and undergo a phenotypic switch consistent with an epithelial-mesenchymal transition (EMT). MDCK cells expressing a truncated mutant of Panx1 exhibited a similar EMT-like phenotypic change and additionally failed to polarize. Taken together, our data indicate that polarized trafficking of Panx1 does not depend upon the dileucine motif, and that the expression of Panx1 mutants may play a role in dysregulating the epithelial cell phenotype.

Keywords

Pannexin, channel, membrane, glycoprotein, pannexin 1 (Panx1), non-polarized, polarized, polarization, epithelial cell, tight junction, Madin-Darby Canine Kidney (MDCK), apical, basolateral, trafficking, targeting, epithelial-mesenchymal transition (EMT), confocal laser microscopy

Co-Authorship Statement

All experiments were performed by Michelle Shum; however, Dr. Cindy Qing Shao was instrumental in generating or obtaining Panx1 constructs and provided technical expertise throughout the project.

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To my family for their unconditional love and support. Thank you for standing by me, regardless of what I pursue.

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Δ379 – Truncation starting at amino acid position 379

AP-2 – Adaptor protein 2

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

Ca^{2+} – Calcium

CME – Clathrin-mediated endocytosis

COPII – Coat protein complex II

DMEM – Dulbecco's modified Eagle's medium

ECM – Extracellular matrix

EL1 – Extracellular loop 1

EL2 – Extracellular loop 2

EMT – Epithelial-to-mesenchymal transition

ER – Endoplasmic reticulum

FBS – Fetal bovine serum

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GFP – Green fluorescent protein

GPI - Glycosylphosphatidylinositol

HCl – Hydrochloric acid

HEK – Human embryonic kidney

IL – Intracellular loop

kDa – Kilodalton

LL365/6AA – dileucine to dialanine substitution at amino acid positions 365 and 366

MDCK – Madin-Darby canine kidney

mRNA – Messenger RNA

NaCl – Sodium chloride

NaF – Sodium fluoride

NMDA - *N*-methyl-D-aspartate

NRK – Normal rat kidney

PANX1 – Human gene for PANX1

Panx1 – Mouse gene for Panx1

PANX1 – Human Pannexin 1

Panx1 – Pannexin 1

Panx2 – Pannexin 2

Panx3 – Pannexin 3

pS – Picosiemens

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

T307 – Threonine at amino acid position 307

TEER – Transepithelial electrical resistance

WT – Wildtype

Y308 – Tyrosine at amino acid position 308

Y308F – Tyrosine to phenylalanine substitution at amino acid position 308

Chapter 1

1 Introduction

1.1 Pannexin family of channel-forming proteins

Pannexins (Panx1, Panx2 and Panx3) are a family of channel-forming transmembrane glycoproteins that generally reside at the plasma membrane. First identified in the mammalian genome by Panchin and colleagues in the year 2000, pannexins were initially believed to compose a new family of gap junction proteins (Bruzzone et al., 2003; Panchin et al., 2000). Overwhelming evidence now indicates that pannexins form single membrane channels that mediate the release of molecules to the extracellular environment (Scemes et al., 2009; Sosinsky et al., 2011). Topologically, pannexins consist of four alpha-helical transmembrane domains, two extracellular loops (EL1 and EL2), one intracellular loop (IL) and both the N- and C-termini exposed to the cytoplasm (**Fig. 1.1**) (Baranova et al., 2004). Whereas the N-terminus of each pannexin is well-conserved between family members, the highest degree of sequence variability resides in the C-terminal tails, with Panx1 and Panx3 being the most homologous to one another out of the three subtypes (Baranova et al., 2004; Penuela et al., 2007). Similar to the connexin family of gap junction proteins, individual Panx1 channels contain 6 subunits, while Panx2 channels have been reported to be octameric (**Fig. 1.1**) (Ambrosi et al., 2010; Boassa et al., 2007). The oligomerization status of Panx3 remains undefined, but is predicted to be hexameric due to its homology with Panx1 (Penuela et al., 2013; Scemes & Velíšková, 2017).

Pannexin channels function as conduits for the release of small molecules and ions from the cell to the extracellular milieu. Molecules that pass through pannexin channels must conform to limitations on the physical shape, size and charge of the molecule, depending on the properties of the pannexin channel pore. For Panx1, the pore-lining residues near the outer leaflet of the membrane reside on the first transmembrane domain as well as EL1 (Wang & Dahl, 2010). Electron microscopic studies reveal that Panx1 channel pores may take on two measurements, either ~17 or 21 Å (Ambrosi et al., 2010). In addition, there are conflicting reports regarding the single membrane conductance of active Panx1 channels; although

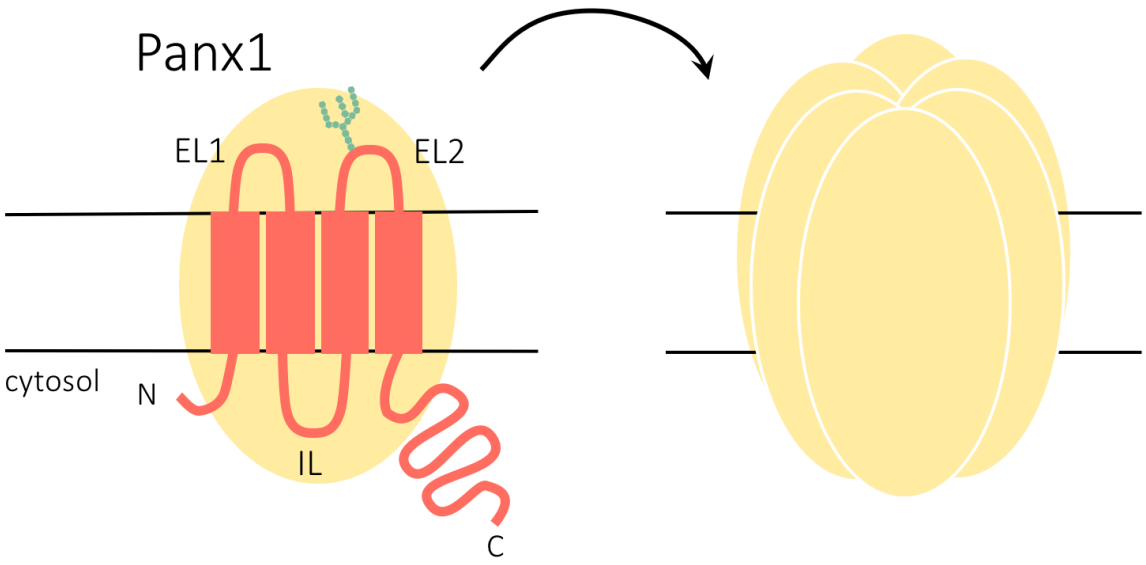

Figure 1.1

Figure 1.1. Pannexins are channel-forming membrane glycoproteins. Pannexins are polytopic membrane glycoproteins consisting of four transmembrane domains, two extracellular loops, one intracellular loop and cytoplasmic N- and C-terminal domains. Single membrane channels form when pannexin monomers oligomerize. Depicted is Panx1, which is believed to form hexameric channels. Panx1 is glycosylated at N254 of EL2 (green moiety). EL = extracellular loop; IL = intracellular loop.

initially reported to be ~500 pS (Bao et al., 2004; Wang & Dahl, 2010), Panx1 channels are now believed to have conductances of ~100 pS (Chiu et al., 2017). Pannexin family members are able to intermix with one another (Ambrosi et al., 2010; Bruzzone et al., 2005; Penuela et al., 2009). In particular, Panx1 and Panx2 are known to interact in a glycosylation-dependent manner that negatively affects the ability of the channels to mediate dye uptake compared with Panx1 homomeric channels (Penuela et al., 2009). Panx1 and Panx3 also intermix; however, this interaction may be weaker than that between Panx1 and Panx2, and does not appear to affect channel function (Penuela et al., 2009). The oligomeric status of such pannexin heteromers are yet to be determined, as these mixed channels are unstable over time (Ambrosi et al., 2010). Nevertheless, the ability of pannexins to potentially co-oligomerize *in vivo* expands the breadth of possible channel subtypes and cellular functions.

1.1.1 Distribution of pannexins in mammalian tissues

At least one of the three pannexin family members is expressed in virtually all mammalian tissues. Panx1 is the most ubiquitously expressed in both human and murine tissues, being found in the skin, brain, spleen, kidney, liver, cartilage, skeletal muscle, blood vessels, immune cells and various parts of the central nervous system and the reproductive system of the mouse (Penuela et al., 2007). Panx2 immunolabeled protein has been detected in tissues such as the kidney, testes, small intestine, thymus, heart, colon, lung and eye, although its mRNA is predominantly found in tissues of the central nervous system (Diezmos et al., 2015; Le Vasseur et al., 2014). Panx3 is expressed largely in the skin, the male reproductive tract, cartilage and bone (Bond et al., 2011; Celetti et al., 2010; Ishikawa et al., 2011; Iwamoto et al., 2010; Penuela et al., 2007; Turmel et al., 2011).

1.1.2 Subcellular localization profiles of pannexins

Although pannexins are best characterized as large pore channels at the plasma membrane, their distribution at the subcellular level is diverse, strongly suggesting that these channels may acquire alternative functional roles in other compartments of the cell. In addition to the cell surface, Panx1 and Panx3 localize to perinuclear compartments in select cell types, both endogenously and after ectopic expression (Penuela et al., 2008; Penuela et al., 2009). In LNCaP prostate adenocarcinoma cells, exogenously-introduced Panx1 was detected at the cell surface and additionally colocalized with the endoplasmic reticulum (ER) marker, BODIPY-brefeldin A (Vanden Abeele et al., 2006). Similarly, in C2C12 osteoblasts,

endogenous Panx3 has been detected throughout the cell, partially co-distributing with the ER marker calnexin (Ishikawa et al., 2011). The subcellular localization profile of Panx2 in cells is distinctly different from that of Panx1 and Panx3. Panx2 is predominantly localized to intracellular compartments and exhibits limited cell surface distribution, with the exception of ectopic expression systems (Boassa et al., 2015; Penuela et al., 2009; Swayne et al., 2010). The exact function of pannexin channels in these intracellular contexts is poorly understood; however, evidence suggests that, at least for Panx1 and Panx3, ER resident pannexin channels mediate the release of Ca^{2+} ions into the cytoplasm, which modulates cellular processes such as gene expression and cell proliferation (Ishikawa et al., 2011; Vanden Abeele et al., 2006).

1.1.3 Life cycle and post-translational modifications of pannexins

As Panx1 is the most well-studied pannexin family member, much of the literature pertaining to pannexin trafficking applies mainly to this pannexin subtype. Pannexins are first co-translationally inserted into the ER membrane as core (unglycosylated) proteins (gly0). In the ER, all 3 pannexins oligomerize and are *N*-glycosylated to high mannose-containing glycoproteins (gly1) (Penuela et al., 2014). Panx1 is glycosylated at N254 of EL2, while glycosylation of Panx2 occurs at N86 of EL1 (Boassa et al., 2007; Penuela et al., 2007; Sanchez-Pupo et al., 2018). Panx3 is glycosylated at N71 of EL1 (Penuela et al., 2007; Sanchez-Pupo et al., 2018). From the ER, Panx1 and Panx3 are trafficked via Sar1-dependent coat protein complex II (COPII)-coated vesicles to the Golgi apparatus (Bhalla-Gehi et al., 2010). In the Golgi, high mannose-glycosylated forms of Panx1 and Panx3 are further modified into complex glycoproteins, termed the gly2 forms of Panx1 and Panx3, and subsequently trafficked to the plasma membrane (Bhalla-Gehi et al., 2010). Panx2 proteins are not modified into the gly2 form (Penuela et al., 2009). Transport of Panx1- and Panx3-containing vesicles occurs along the actin cytoskeletal network and does not rely on microtubules (Bhalla-Gehi et al., 2010; Wicki-Stordeur & Swayne, 2013).

From the cell surface, pannexin channels are internalized via a mechanism that has not yet been fully defined. For Panx1, internalization does not appear to involve clathrin- or caveolin-mediated endocytic machinery, or the activity of dynamin II, as Panx1 does not co-immunoprecipitate with key proteins involved in any of the aforementioned processes (Gehi et al., 2011). The mechanism of Panx1 endocytosis has been proposed to be cell- and

context-dependent, as there are conflicting reports concerning the dependence of Panx1 endocytosis on cholesterol-rich lipid rafts (Boyce et al., 2015; Gehi et al., 2011). Once inside the cell, Panx1 proteins are sorted to the early endosome, the late endosome and, lastly, the endolysosome before being preferentially degraded in lysosomes. Misfolded or mutated Panx1 proteins appear to be destined for proteasomal degradation (Boyce et al., 2015; Gehi et al., 2011). Panx1 internalization can be triggered by the elevation of extracellular potassium, exogenous ATP and intracellular calcium (Boyce et al., 2015; Boyce et al., 2014). The degradation pathway of Panx2 is unresolved and may also exhibit dependence on the cellular context, as there are contradictory reports regarding whether Panx2 colocalizes with lysosomal compartments (Boassa et al., 2015; Sanchez-Pupo et al., 2018; Wicki-Stordeur et al., 2013).

Pannexins undergo many post-translational modifications in addition to glycosylation (**Fig. 1.2**). Panx1 is phosphorylated at both Y198 and Y308 and is *S*-nitrosylated at C40 and C346 (Lohman et al., 2012, 2015; Weilinger et al., 2016). Phosphorylation of Panx1 at Y308 has been demonstrated to functionally close Panx1 channels, whereas Y198 phosphorylation is postulated to have the same effect (Lohman et al., 2015; Weilinger, Tang, & Thompson, 2012). *S*-nitrosylation is also known to inhibit Panx1 function (Lohman et al., 2012). Increased hydrogen peroxide in the cell has been associated with the formation of disulfide bonds in Panx1 channels, and temporarily inhibits Panx1-induced currents (Krick et al., 2016). Finally, Panx1 is best known to undergo caspase-mediated C-terminal cleavage, which renders Panx1 channels irreversibly active and thus increases membrane permeability during apoptosis (Chekeni et al., 2010; Sandilos et al., 2012). Ubiquitination has been proposed as a possible modification of Panx1, but direct evidence of this alteration is lacking (Johnstone et al., 2012). Modifications of Panx2 and Panx3 are less well-defined. Panx2 has been shown to be palmitoylated (Swayne et al., 2010), while modifications in Panx3 other than glycosylation remain uninvestigated. It should be noted that the Panx2 carboxy tail may act as a substrate for caspase cleavage; however, it is unclear whether this occurs in living systems (Penuela et al., 2014).

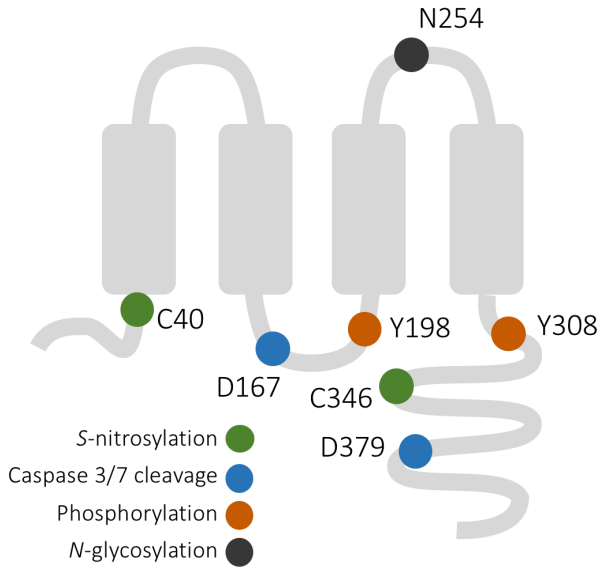
N254
C40
Y198
Y308
D167
C346
D379
S-nitrosylation
Caspase 3/7 cleavage
Phosphorylation
N-glycosylation

Figure 1.2

Figure 1.2. Post-translational modifications of Panx1. With the exception of N254 in EL2, all post-translational modifications of Panx1 are located in the cytoplasmic domains of the protein. Green residues = *S*-nitrosylation sites; blue residues = caspase 3/7 cleavage sites; maroon residues = phosphorylation sites; black residue = *N*-glycosylation site. Cleavage at D167 has only been demonstrated *in vitro*, and it is unknown whether this modification occurs *in vivo*.

1.1.4 Pannexin trafficking motifs

Current understanding of the exact targeting motifs involved in regulating pannexin trafficking is relatively limited. The glycosylation status of Panx1 and Panx3 are crucial to proper cell surface trafficking, as mutation of the glycosylation site of both pannexins causes each protein to be preferentially retained in intracellular compartments (Boassa et al., 2008; Penuela et al., 2009). Interestingly, co-expression of the glycosylation-deficient Panx1 mutant with wild type monomers rescues this mistrafficking (Boassa et al., 2008).

In addition, the C-terminal domain of Panx1 is known to play a crucial role in protein trafficking. Previously, Gehi and colleagues demonstrated that the C-terminal tail downstream of T307 is indispensable to its cell surface delivery, indicating that this region of Panx1 functions as a plasma membrane targeting motif (Gehi et al., 2011). Unlike wild type Panx1, which is degraded in lysosomes, T307-truncated Panx1 was shown to undergo ER-associated degradation in the proteasome, suggesting that this mutation affects protein folding and/or oligomerization (Gehi et al., 2011). Intriguingly, fusing the Panx1 C-terminal domain to the channel domain of Panx2 results in a Panx2-Panx1 chimeric protein that exhibits predominantly intracellular localization, demonstrating that the Panx1 C-terminus is not solely sufficient to redirect trafficking to the plasma membrane (Wicki-Stordeur et al., 2013). Overall, the exact motifs involved in pannexin trafficking remain poorly understood.

1.2 Functions of pannexin channels

As large pore conduits for the passage of molecules and ions, pannexin channels are heavily implicated in cell-cell communication. Of all molecules known to pass through these channels, ATP release via Panx1 during cellular apoptosis is the most well-characterized phenomenon. In this context, Panx1 has been shown to be a molecular constituent of the $P2X_7$ death complex, a purinergic receptor complex at the cell surface, which, upon binding of its ligand, will activate a signaling cascade, ultimately leading to Panx1 channel opening and release of ATP (Locovei et al., 2007; Pelegrin & Surprenant, 2006). This nucleotide release from apoptotic cells has been documented to recruit activated phagocytes (Chekeni et al., 2010; Sandilos et al., 2012). Another function mediated by ATP release from Panx1 is the propagation of calcium waves. When ATP binds to P2Y purinergic receptors at the cell surface, Ca^{2+} stores in the ER are released into the cytoplasm. This increase in cytoplasmic

Ca^{2+} activates Panx1 channels (Locovei et al., 2006), which leads to further ATP release and initiation of Ca^{2+} waves in neighbouring cells (Penuela et al., 2013). This propagation is proposed to occur notably in erythrocytes, whereby the release of ATP initiates Ca^{2+} waves in endothelial cells (Locovei et al., 2006). The resultant increase in cytosolic Ca^{2+} ultimately leads to NO production, which signals to smooth muscle cells to initiate vasodilation (Locovei et al., 2006). During HIV-1 infection, Panx1 channels are activated upon binding of viral envelope proteins to cell receptors. The subsequent release of ATP indirectly causes membrane depolarization and leads to fusion of the virion with the target cell membrane (Orellana et al., 2013; Séror et al., 2011). ATP signaling mediated by Panx1 is additionally involved in taste sensation, keratinocyte differentiation and neuronal excitotoxicity (Celetti et al., 2010; Dando & Roper, 2009; Penuela et al., 2013; Weilinger et al., 2016).

In addition to ATP, the pro-inflammatory cytokine interleukin-1β has been documented to pass through Panx1 channels (Pelegrin & Surprenant, 2006). This function is activated downstream of the $P2X_7$ receptor and was shown to be crucial for the processing of caspase-1 by the inflammasome in macrophages, neurons and astrocytes (Pelegrin & Surprenant, 2006; Silverman et al., 2009).

Much less is known regarding the cellular functions of Panx2 and Panx3. Panx2 has been shown to play a role in neuronal differentiation (Swayne et al., 2010), but its role in other tissues is less clear. Panx3 has been implicated in keratinocyte differentiation, chondrogenic and osteogenic differentiation, as well as odontoblast proliferation and differentiation (Bond et al., 2011; Celetti et al., 2010; Iwamoto et al., 2010, 2017).

1.2.1 Channel-independent functions of pannexins: the interactome

In addition to signaling via release of molecules into the extracellular space, pannexins additionally possess channel-independent functions in the cell. Investigation into the pannexin interactome affords the opportunity to consider the possible roles of pannexins that are not directly linked to its channel function. A proteomics analysis of Panx1-interacting proteins revealed that Panx1 interacts extensively with other cell surface receptors, ion channels, their signaling complexes and components of the cytoskeleton (Wicki-Stordeur & Swayne, 2013, 2014). Of notable significance is the interaction between the Panx1 C-terminal domain and actin, which has been consistently shown across different cell types

(Gehi et al., 2011; Wicki-Stordeur & Swayne, 2013). This interaction suggests that Panx1 may play a role in cellular processes that involve microfilaments, such as cell migration, cell morphogenesis and vesicular trafficking. Indeed, ectopically expressed Panx1 in BICR-M1R$_k$ cells was found to be particularly enriched in areas of the plasma membrane that correlate with invadopodia (Bhalla-Gehi et al., 2010). This was similarly observed in BICR-M1R$_k$ cells expressing exogenous Panx3 (Bhalla-Gehi et al., 2010). Finally, in N2a cells, siRNA-mediated knockdown of Panx1 was associated with reduced cell migration (Wicki-Stordeur & Swayne, 2013). As discussed earlier, pannexins have also been shown to interact with other family members; specifically, Panx1 and Panx2, as well as Panx1 and Panx3 (Penuela et al., 2009). However, whether these intermixed oligomers form *in vivo* remains unclear and is questionable, given that Panx1/Panx2 heteromers are unstable over time (Ambrosi et al., 2010).

1.3 Pannexins in disease contexts

Considering the diversity of tissues in which pannexins are expressed, it is not surprising that pannexins have been implicated in over a dozen human pathologies, most of which have been studied with regard to the involvement of Panx1 (Penuela et al., 2014). Many pannexin-linked diseases involve multiple tissue types and support the general view that suppression of pannexin protein expression and/or channel activity can be protective against disease progression, although several exceptions exist.

Although its exact role in cancer appears to be tissue-specific, the contribution of pannexins to cancer progression has been investigated. In glioma cells, Panx1 and Panx2 have been found to be downregulated compared to primary astrocytes, indicating that both pannexins may act as tumor suppressors in this capacity (Lai et al., 2009; Lai et al., 2007). In contrast, a study by Furlow and colleagues showed that expression of a hyperactive gain-of-function Panx1 truncation mutant (Panx1$^{1-89}$) was linked to increased aggressiveness and augmented metastatic potential of breast cancer cells (Furlow et al., 2015). Furthermore, Penuela and colleagues have previously demonstrated that downregulation of Panx1 in melanoma cells leads to a less invasive and more differentiated melanocytic phenotype (Penuela et al., 2012). Collectively, our knowledge regarding the role of pannexins in cancer remains incomplete, but likely involves differences in the tissue and tumor microenvironment, as well as the complex interplay between neighbouring cells and the surrounding stroma.

In the central nervous system, Panx1 expression is generally associated with cell death, notably in neurons challenged with environmental insults such as hypoxia and glucose deprivation (Thompson et al., 2006). Panx1 channels in neurons form a signaling complex that is activated upon stimulation of *N*-methyl-D-aspartate (NMDA) receptors, and pathological activation of this complex leads to excessive Panx1 channel activity that is associated with seizure-like episodes of neuronal firing (Thompson et al., 2008). This phenotype is partially rescued upon either pharmacological inhibition or genetic deletion of Panx1 (Santiago et al., 2011).

In the colon, a reduction in Panx1 expression was observed in patients suffering from ulcerative colitis and Crohn's disease (Diezmos et al., 2013). This decrease in Panx1 protein expression was attributed to a loss of fully functional enteric neurons, leading to the postulation that ATP release via Panx1 channels in the colonic epithelium is involved in sensory transduction and secretion of nutrients into the luminal space (Deizmos et al., 2013).

Pannexins are additionally involved in pathological conditions such as glaucoma, osteoarthritis, hypertension and infectious diseases such as HIV (Orellana et al., 2013; Penuela et al., 2014; Séror et al., 2011). A 2016 report from the Laird laboratory describing an individual born with a germline mutation in the *PANX1* gene was the first to document a case of an inherited "*PANX1*-related disorder" (Shao et al., 2016). This patient exhibited widespread organ dysfunction that appeared to be recessively inherited. Moreover, this mutant was found to be a loss-of-function Panx1 variant, strongly suggesting that reduced Panx1 function severely impairs tissue homeostasis. Overall, the role of pannexins in disease contexts are just beginning to emerge, and understanding the biology of pannexin regulation and function will undoubtedly uncover new therapeutic considerations.

1.4 Polarized epithelial cells

Epithelial cells are the cells that collectively line the surfaces and cavities of the body. Epithelia can take on several morphological forms; in simple epithelia, epithelial cells are arranged as a monolayer in which each cell maintains contact with the underlying basement membrane. In stratified epithelia, cells of the epithelium form multiple layers, in which only cells of the basal layer are anchored to the basement membrane.

Epithelial cells are tasked with the function of simultaneously protecting the organism and facilitating appropriate exchange of nutrients between the organism and the luminal environment, according to cellular and tissue requirements (Macara et al., 2014). As a result, these cells must form specialized plasma membrane domains to mediate selective absorption and secretion across the epithelium. The cell surface of simple epithelial cells is thus compartmentalized into distinct domains: the apical membrane domain, which faces toward the lumen or external environment; and the basolateral membrane domain, which refers to the portion of the plasma membrane that faces the connective tissue. The functional asymmetry in protein and lipid composition of the epithelial cell plasma membrane is referred to as the polarity of the epithelial cell (Mostov et al., 2000).

1.4.1 Tight junctions maintain cell surface polarity

The apical and basolateral cell surfaces are physically segregated by intercellular adhesion complexes called tight junctions (**Fig. 1.3**) (Zihni et al., 2016). In addition to adhering neighbouring cells at the apical-lateral membrane, tight junctions serve two more functions: first, they form semi-permeable gates along the paracellular pathway to limit the passage of molecules between cells; and second, tight junctions restrict the diffusion of integral membrane proteins between the apical and basolateral membrane compartments (Zihni et al., 2016). This second function of tight junctions is essential to maintaining the cell surface polarity of epithelial cells. Tight junctions are situated along the lateral membrane between adjacent epithelial cells, in close proximity to the apical portion of the cell, representing the most apical component of all intercellular adhesion complexes in epithelial cells. The roles of tight junctions in mediating intracellular signaling and even vesicular docking are continually emerging, indicating that the function of this adhesion complex is more dynamic than previously believed (Balda & Matter, 2009; Zihni et al., 2016).

Tight junctions assume the arrangement of long strands of interacting membrane proteins that span the entire circumference of each epithelial cell. Compositionally, tight junctions are well-defined; the main transmembrane components are claudins, occludins and junctional

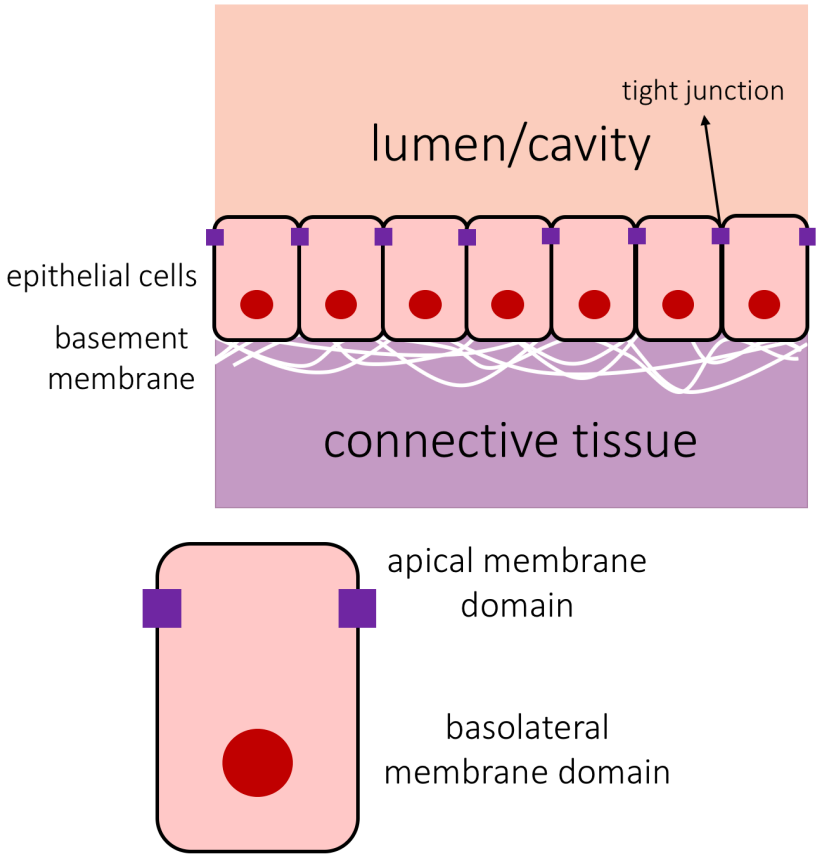
tight junction
lumen/cavity
epithelial cells
basement membrane
connective tissue
apical membrane domain
basolateral membrane domain

Figure 1.3

Figure 1.3. Tight junctions form the boundary between the apical and basolateral membrane domains of polarized epithelial cells. Adjacent cells in the epithelium are joined laterally by complexes of interacting membrane proteins called tight junctions. Tight junctions limit the passage of molecules between cells and act as a fence to restrict diffusion of membrane proteins across the junction. This creates two distinct plasma membrane compartments: the apical and the basolateral membrane domains, which face toward and away from the lumen, respectively.

adhesion molecules (JAMs). Most tight junctions in epithelia form between two adjacent cells (bicellular junction); however, tight junctions are also known to exist at corners in which three cells converge (Ikenouchi et al., 2005). In these tricellular contacts, a fourth transmembrane protein, tricellulin, can be found (Ikenouchi et al., 2005). Claudins are the main determinant of paracellular tight junction permeability (Gunzel & Yu, 2013). On the cytoplasmic face of tight junctions are adaptor proteins, collectively forming the junctional plaque, which mediate interaction of tight junction components with intracellular protein networks, such as the actin cytoskeleton (Zihni et al., 2016). Of all known adaptor proteins, zonula occludens (ZO) proteins (ZO-1, ZO-2 and ZO-3) exemplify the prototypical tight junctional plaque proteins, though ZO-1 is also known to interact significantly with gap junctions and adherens junctions at the basolateral cell surface (Palatinus et al., 2010; Toyofuku et al., 1998; Zihni et al., 2016).

1.4.2 Polarized protein trafficking in epithelial cells

Protein trafficking and vesicular transport in epithelial cells require complex regulatory mechanisms in order to maintain cell surface polarity. Apical- and basolateral-specific trafficking routes are thus established in polarized epithelial cells. Two general transmembrane protein trafficking pathways have been identified for transmembrane proteins in polarized cells: the direct and indirect routes (**Fig. 1.4**) (Mostov et al., 2000). In the direct pathway, proteins are sorted directly from the Golgi to the apical or basolateral membrane domain. The indirect pathway involves transcytosis; proteins are initially trafficked to one membrane surface (usually basolateral, in the case of polarized Madin-Darby canine kidney (MDCK) cells), and subsequently endocytosed and delivered to early endosomes (Apodaca et al., 1994; Mostov et al., 2000). In MDCK cells, this is generally the basolateral early endosome. Subsequently, proteins retrieved from the basolateral domain are further sorted into the common endosome before reaching the recycling endosome (apical recycling endosomes for apically-destined proteins). Lastly, recycling endosomes deliver their cargo to a different domain of the plasma membrane (mainly the apical domain in MDCK cells) (Apodaca et al., 1994; Mostov et al., 2000). Collectively, this process of retrieval and delivery of membrane proteins from one domain to the other is called transcytosis (Fung et al., 2017).

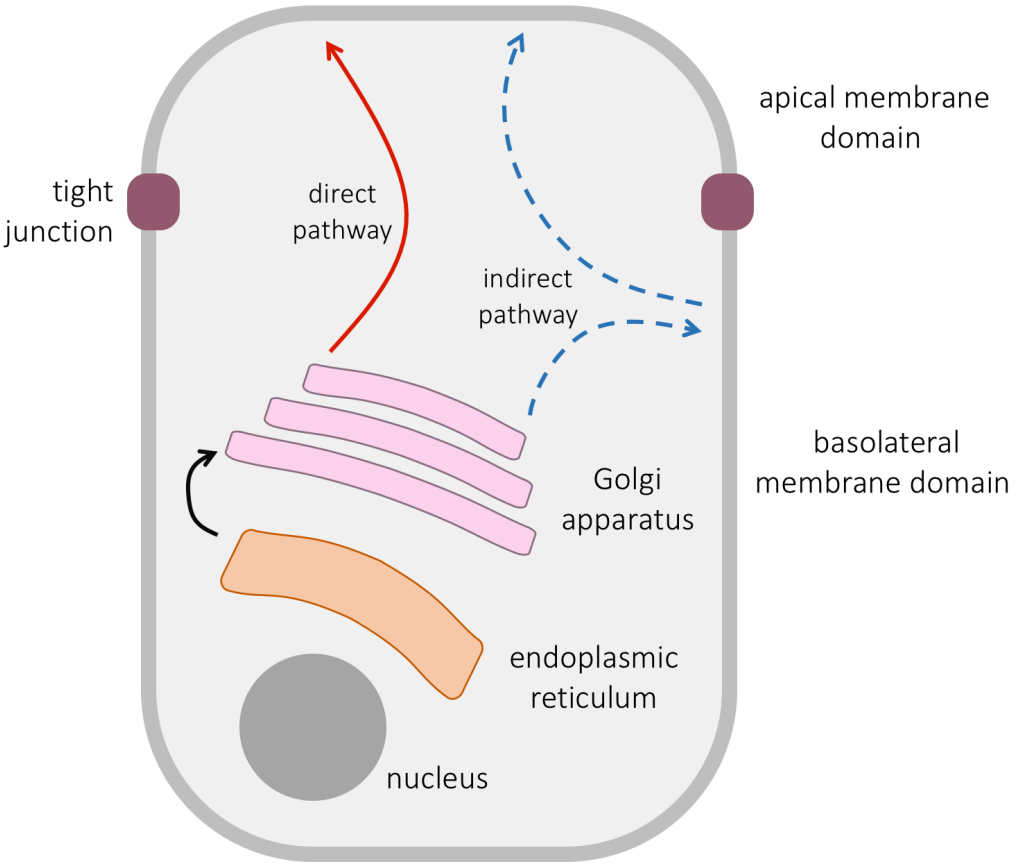
apical membrane
domain
tight
junction
direct
pathway
indirect
pathway
basolateral
membrane domain
Golgi
apparatus
endoplasmic
reticulum
nucleus

Figure 1.4

Figure 1.4. Trafficking pathways for apically-destined proteins in polarized epithelial cells. Proteins destined for the apical membrane domain are initially transported from the ER to the Golgi. In the direct pathway, proteins are immediately trafficked to the apical membrane from the Golgi (red arrow). The indirect or transcytotic route involves first transporting the protein to the basolateral surface before endocytosis, after which the protein is trafficked to the apical cell surface (blue dashed arrows).

Discrete peptide motifs on the cytoplasmic domain of transmembrane proteins typically direct protein trafficking to the basolateral cell surface (Mostov et al., 2000; Stoops & Caplan, 2014). The most thoroughly defined sorting motifs for basolaterally-destined proteins are tyrosine-based (NPXY and YxxØ, in which Ø represents a large hydrophobic amino acid residue) and dileucine-based (D/ExxxLL) (Stoops & Caplan, 2014). In contrast, apical sorting motifs are less well-defined and may be found in any domain of apically-destined proteins (Mostov et al., 2000; Stoops & Caplan, 2014). In general, glycosylphosphatidylinositol (GPI)-anchors and oligosaccharide modifications of proteins have been shown to target proteins to the apical domain, although not always sufficient (Mostov et al., 2000).

1.5 Pannexins in polarized epithelial cells

Reports of the *in vivo* localization of Panx1 in polarized epithelial cells indicate that Panx1 protein distribution may be tissue-dependent. For example, Panx1 is documented to localize to the apical surface of cells of the respiratory epithelium (Ransford et al., 2009); yet, in the rat epididymis, Panx1 has been detected at the basolateral interface of each epithelial cell (Turmel et al., 2011). In the kidney, Panx1 expression was enriched at the apical surface of cells lining the loop of Henle and the collecting duct (Hanner et al., 2012). In the colon, Panx1 expression was restricted to the basolateral plasma membrane (Le Vasseur et al., 2014). Conversely, Panx1 was detected throughout the apical and basolateral cell surfaces of umbrella cells in the rat urothelium (Beckel et al., 2015).

In this report, we focus our investigation solely on localizing Panx1 for a number of reasons; (1) Panx1 is the most widely expressed pannexin throughout the human body; (2) the trafficking route and cellular dynamics of Panx1 in non-polarized cells are the most well-characterized amongst the three pannexin family members; and (3) Panx1 is the predominant pannexin subtype found at the plasma membrane of epithelial cells *in vivo*.

1.6 Modeling pannexins in polarized epithelial cells

It is imperative to assess the complex regulation of Panx1 trafficking and distribution in polarized epithelial cells, as this will provide valuable insight into its putative functional roles. This is further emphasized when considering differences in Panx1 distribution between

polarized and non-polarized cell types (which may be uncertain due to questionable anti-Panx1 antibody specificity). In non-polarized cells, such as normal rat kidney (NRK) cells, BICR-M1R$_k$ rat mammary tumor cells and human embryonic kidney (HEK) 293T cells, Panx1 is found throughout the plasma membrane (Penuela et al., 2007, 2013). In the case of BICR-M1R$_k$ cells, Panx1 was found to localize to multiple sites, both within intracellular compartments and throughout the cell surface, including various plasma membrane protrusions (Bhalla-Gehi et al., 2010). In contrast, Panx1 acquires a polarized distribution in most epithelial cells *in vivo* (Hanner et al., 2012; Le Vasseur et al., 2014; Ransford et al., 2009; Turmel et al., 2011). Additionally, whether the Panx1 polypeptide contains any functional sorting motifs targeting it to specific membrane domains is unknown. Nevertheless, it is clear from *in vivo* localization studies that Panx1 trafficking in polarized epithelial cells is regulated in a manner that is distinct from that of non-polarized cells.

To assess the regulation of protein distribution in polarized epithelial cells, many investigators utilize MDCK cells as a versatile *in vitro* model of polarized cells (Leighton et al., 1970; Shamir & Ewald, 2015). In two-dimensional cell culture, these cells are maintained in a non-polarized state, in which they do not generally exhibit tight junction formation, and thus no separation of the cell surface into apical or basolateral compartments (Leighton et al., 1970). When grown on porous membranes, where they are fed from above and below the cell monolayer, or in extracellular matrix (ECM), MDCK cells form sheets and cysts of polarized cells, respectively. A small number of studies have used MDCK cells as a host cell to investigate various aspects of Panx1 biology; however, none have assessed the role of MDCK cell polarization on the distribution of Panx1 (Boassa et al., 2007, 2008, 2015; Penuela et al., 2008). In these studies, MDCK cells were used largely to investigate the effects of oligomerization and glycosylation of Panx1. Due to this gap in knowledge of how Panx1 is sorted in polarized epithelial cells, we chose to use the MDCK cell line in order to investigate the trafficking and distribution of Panx1 in polarized and non-polarized cells *in vitro*.

1.7 Hypothesis

We hypothesized that Panx1 is differentially distributed in polarized cells versus non-polarized cells, and that this selective trafficking is governed by motifs encoded within the Panx1 polypeptide. To address this hypothesis, we ectopically expressed Panx1 in BICR-

$M1R_k$ cells and in MDCK cells to investigate its cellular residency in polarized and non-polarized cells. In addition, we analyzed the amino acid sequence of Panx1 and generated mutations in regions along the C-terminal domain that conform to predicted basolateral-specific sorting motifs (Y308F and LL365/6AA). We also obtained a C-terminal truncation mutant of Panx1 (Δ379) and expressed all mutants in MDCK cells to potentially identify the effect of each mutation on Panx1 trafficking and MDCK cell polarity.

1.8 Objectives

The specific objectives of this study were:

1 To assess the distribution and localization of Panx1 in polarized and non-polarized cells *in vitro*

2 To determine the role of the dileucine peptide motif, the tyrosine-based peptide motif, and C-terminal truncation in targeting Panx1 to specific cell membrane domains of polarized cells

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Chapter 2

2 Localization and trafficking of pannexin 1 in polarized and non-polarized cells

The channel-forming membrane protein pannexin 1 (Panx1) is best characterized as an ATP release channel and has been linked to over a dozen human pathologies. Along with protein-protein interactions and post-translational modifications, the physiological function of Panx1 channels is highly dictated by its subcellular localization. In contrast to non-polarized cell types, Panx1 has been reported to selectively localize to specific membrane compartments in polarized epithelial cells *in vivo*. However, it is not known whether this trafficking is governed by motifs in the Panx1 polypeptide. Here, we investigated the distribution of moxGFP-tagged Panx1 in polarized MDCK cells as well as in non-polar BICR-M1R_k tumor cells. Although Panx1 was detected predominantly at the apical membrane domain of polarized MDCK cells, a small population of Panx1 remained localized to the basolateral cell surface. In non-polarized cells, Panx1 localized throughout the entire plasma membrane, including the lamellipodia of the tumor cells. The distribution of Panx1 remained unchanged upon mutation of a dileucine motif in the C-terminal domain in both cell types, indicating that this motif is not functional in basolateral Panx1 targeting. Stable expression of a Panx1 mutant in which a putative tyrosine-based sorting motif was eliminated caused the cells to lose the ability to polarize and undergo a phenotypic switch consistent with an epithelial-mesenchymal transition (EMT). MDCK cells expressing a truncated mutant of Panx1 exhibited a similar phenotypic EMT-like change and also failed to polarize. Collectively, these results indicate that trafficking of Panx1 in polarized cells does not depend upon the dileucine motif, and that the expression of Panx1 mutants may play a role in dysregulating the epithelial cell phenotype.

2.1 Introduction

Pannexins (Panx1, Panx2, Panx3) are a family of large-pore membrane glycoproteins that allow the passage of molecules and ions from the cell to the extracellular space (Panchin et al., 2000). Topologically, pannexins contain four transmembrane domains that are linked by two extracellular loops, one intracellular loop, and N- and C-terminal domains facing the cytoplasm (Baranova et al., 2004). Of the three subtypes, Panx1 represents the most widely expressed and well-characterized family member, and is known to function as an ATP release channel (Bao et al., 2004; Chekeni et al., 2010; Locovei et al., 2006; Penuela et al., 2013; Séror et al., 2011). ATP release by Panx1 is involved in various cellular processes such as apoptosis, Ca^{2+} wave propagation, and inflammasome activation (Chekeni et al., 2010; Locovei et al., 2006; Pelegrin & Surprenant, 2006; Silverman et al., 2009). Dysregulation of Panx1 channel activity and/or expression is frequently associated with pathologies (Dvoriantchikova et al., 2012; Freitas-Andrade et al., 2017; Jiang & Penuela, 2016; Penuela et al., 2013; Penuela, et al., 2014; Weilinger et al., 2016). Recently, a germline mutation in *PANX1* was also found in a patient with extensive multisystem defects, further enforcing the significance of proper Panx1 function on cell and tissue homeostasis (Shao et al., 2016).

Trafficking of Panx1 occurs via the classical endoplasmic reticulum (ER)-Golgi exocytic pathway (Bhalla-Gehi et al., 2010). After being translated and inserted into the ER membrane, Panx1 proteins oligomerize into hexameric channels that are transported to the Golgi apparatus via Sar1-dependent COPII-coated vesicles (Bhalla-Gehi et al., 2010). From the Golgi, Panx1 channels are typically delivered to the plasma membrane, where they exert their function as mediators of autocrine and paracrine signaling. Cell surface-localized Panx1 channels are then internalized and degraded in lysosomes (Gehi et al., 2011). Both *N*-linked glycosylation and the C-terminal domain are necessary for cell surface expression of Panx1, suggesting that delivery of Panx1 to the cell surface is a highly regulated process (Boassa et al., 2007, 2008; Gehi et al., 2011; Penuela et al., 2007).

In polarized epithelial cells, where the cell surface is compartmentalized into apical and basolateral membrane domains, Panx1 has been documented to localize to either or both

domains in a tissue-dependent manner (Hanner et al., 2012; Le Vasseur et al., 2014; Ransford et al., 2009; Turmel et al., 2011). A number of studies have reported that Panx1 resides in the apical portions of the respiratory epithelium, the loop of Henle and the collecting duct of the murine kidney (Hanner et al., 2012; Ransford et al., 2009; Turmel et al., 2011). In contrast, Panx1 has been detected largely at the basolateral cell surfaces of epithelial cells in the epididymis and the colon (Le Vasseur et al., 2014; Turmel et al., 2011). Collectively, these data would suggest that Panx1 has sorting motifs encoded within its amino acid sequence that regulates its fate in polarized cells. However, the motifs involved in the polarized trafficking of Panx1 have yet to be investigated. Two well-defined sorting motifs have been identified in many basolaterally-destined proteins: a tyrosine-based motif (YxxØ) and a dileucine-based motif (D/ExxxLL) (Mostov et al., 2000; Stoops & Caplan, 2014). Apical sorting motifs are generally more diverse in nature, and may take on the form of GPI anchors, glycosylation moieties and less well-defined amino acid motifs (Stoops & Caplan, 2014).

In this study, we examined the distribution and localization of Panx1 in polarized and non-polarized cells using *in vitro* models. We additionally assessed the role of the tyrosine-based motif, the dileucine motif, and C-terminal truncation on the distribution of Panx1 in polarized cells. Briefly, Panx1 acquired a predominantly apical distribution profile upon polarization of MDCK cells, although a small population was still detectable at the basolateral cell surface. Basolateral-localized Panx1 was sorted independently of the dileucine motif. Lastly, mutation of the tyrosine-based motif, as well as C-terminal truncation of Panx1, caused widespread cellular changes resulting in MDCK cells acquiring a more mesenchymal-like phenotype.

2.2 Materials and methods

2.2.1 Cell culture and reagents

Madin-Darby canine kidney (MDCK) II cells and HeLa cells were purchased from ATCC (Manassas, VA, USA). The BICR/M1R$_k$ line of rat mammary tumor cells was a gift from Dr. Dieter F. Hülser (University of Stuttgart, Stuttgart, Germany). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose (Cat #11960-044, Life Technologies, CA, USA) supplemented with 10% fetal bovine

serum (FBS) (Cat #098150, Wisent Bioproducts, QC, Canada), 100 units/mL penicillin-streptomycin (Cat #15140-122, Life Technologies) and 2 mM L-glutamine (Cat #25030-081, Thermo Fisher Scientific, MA, USA). Cells were maintained at 37°C and 5% CO_2. To polarize MDCK cells as a monolayer, cells were seeded at a density of 2.0×10^5 cells per 3.0 μm pore-size 24-well Transwell insert (Cat #353492, BD Falcon, NY, USA). Inserts were pre-coated with 50 μg/mL type I rat tail collagen (Cat #354236, Corning Life Sciences, NY, USA). Cells were maintained in culture with daily medium changes until polarized. Cell polarity was monitored by daily measurement of transepithelial electrical resistance (TEER). The resistance ($\Omega \times cm^2$) was obtained by multiplying the sample resistance by the effective area of the Transwell membrane (0.33 cm^2). MDCK cell spheroids were grown as previously described (Engelberg et al. 2011). Briefly, cells were seeded as a suspension in 2% Matrigel (Cat #356234, Fisher Scientific) into 35 mm Ibidi μ-Dishes (Cat #81156, Ibidi) precoated with 50% Matrigel at a density of 6.0×10^2 cells per dish. Spheroids were cultured for 1 week before fixation.

2.2.2 Expression constructs and transfections

The gene encoding Panx1 in human is referred to as *PANX1*, whereas in mice, the orthologous gene is referred to as *Panx1*. *PANX1*-encoding constructs were purchased from InvivoGen (pUNO1-hPanx1) and used as a positive control in Fig 2.1. Expression constructs encoding moxGFP-tagged mouse Panx1 were kindly provided by Dr. Patrick Lajoie and Dr. Silvia Penuela (The University of Western Ontario, London, ON, Canada) (Penuela et al., 2007). The generation and sequence verification of the Y308F, LL365/6AA and Δ379 Panx1-moxGFP mutant constructs were performed via special order to NorClone Biotech Laboratories (London, ON, Canada). All mutant constructs were based on the mouse Panx1 coding sequence.

Transient transfections were carried out using Mirus TransIT-LT1 Transfection Reagent (Cat #MIR2300, Mirus Bio LLC, WI, USA) and Lipofectamine 2000 (Cat #11668027, Thermo Fisher Scientific). Cells were grown to 60-70% confluence in 60 mm culture dishes and transfected with 2 μg of plasmid DNA purified using QIAGEN Maxiprep Kit (Cat #12162, Qiagen, Hilden, Germany). Cells were fixed or harvested 24 to 48 hours

following transfection. Multiclonal stable cell lines were generated by selection with 0.5 mg/mL G418 Sulfate (Cat #450-130, Wisent Bioproducts) for at least two weeks.

2.2.3 Antibodies and immunofluorescence

Cells cultured on coverslips were fixed for 30 minutes at room temperature with 10% neutral-buffered formalin, permeabilized in 0.2% Triton X-100 for 30 minutes and blocked for 30 minutes in 3% bovine serum albumin (BSA). Primary antibodies specific to the C-terminus of PANX1 were generated as previously described (Penuela et al., 2007). Samples were incubated overnight at 4°C with primary antibody with the following dilutions: 1/500 rabbit anti-PANX1, 1/100 rabbit anti-calnexin (Cat #AB75801, Abcam), 1/500 rabbit anti-β-catenin (Cat #19807P, Cell Signaling Technology, MA, USA), 1/1000 mouse anti-E-cadherin (Cat #610182, BD Biosciences) and 1/1000 mouse anti-N-cadherin (Cat #610920, BD Biosciences). The secondary antibody used was 1/500-diluted goat anti-rabbit Alexa Fluor 555 (Cat #A21430, Life Technologies).

Transwell and spheroid cultures were fixed for 30 minutes in 10% neutral-buffered formalin, permeabilized for 1 hour in 0.2% Triton X-100 and blocked with 3% BSA for 1 hour. Cells were labeled with the following primary antibodies overnight at 4°C: 1/500-diluted rabbit anti-β-catenin (Cat #19807P, Cell Signaling Technology) and 1/100-diluted mouse anti-occludin (Cat #33-1500, Invitrogen, CA, USA). Secondary antibodies were incubated for 2.5 hours at room temperature using the following dilutions: 1/250 goat anti-rabbit Alexa Fluor 555 (Cat #A21428, Molecular Probes, CA, USA) and 1/250 goat anti-mouse Alexa Fluor 633 (Cat #A21052, Molecular Probes). For all samples, F-actin was stained with 200-fold diluted Alexa Fluor 568 phalloidin (Cat # 12380, Molecular Probes) and nuclei were counterstained using Hoechst 33342 (Cat #H3570, Molecular Probes). Cells were mounted on glass slides with Airvol and imaged using a Zeiss LSM 800 confocal microscope with Airyscan. Images were taken using 40X and 63X objective lenses. Polarized cell monolayers and spheroids were imaged as z-stacks.

2.2.4 Immunoblotting

Cells were lysed using a sodium dodecyl sulfate (SDS)-based extraction buffer containing 1% SDS in 10 mM Tris HCl, pH 7.4, 100 mM NaF, 100 mM sodium orthovanadate and one tablet of Complete Mini proteinase inhibitor mini-EDTA (Cat #11836153001, Roche Diagnostics). Protein quantification was performed using a bicinchoninic acid assay kit (Cat #23225, Thermo Scientific). A total protein amount of 40 µg were resolved on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes using the iBlot dye transfer system. Membranes were blocked for 1 hour in 3% BSA in PBS-Tween 20 (PBST) at room temperature and probed with the following primary antibodies overnight at 4°C at the indicated dilutions: 1/1000-diluted rabbit anti-PANX1 and 1/5000-diluted mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat #MA374, Millipore). Membranes were washed in PBST and incubated with 1/10000 goat anti-mouse Alexa Fluor 680 (Cat #A21057, Life Technologies) and 1/10000 goat anti-rabbit IRDye 800 (Cat #611-132-002, Rockland Immunochemicals, PA, USA) for 45 minutes at room temperature. Blots were visualized on an Odyssey infrared imaging system (LiCor).

2.2.5 Data analyses and software

Pixel intensity plots were generated using the BAR plugin on Fiji. To calculate the percentage of Panx1 fluorescent signal in the apical and basolateral membrane domains, fluorescence intensities (FI) were measured in each membrane domain using ImageJ software. FI in each domain was thresholded by subtracting the FI of the cytoplasmic area. The apical and basolateral membrane domains were defined as the regions of the cell surface situated above and below the tight junction, respectively. The proportion of signal in each membrane domain was calculated using the following formula:

$$Apical\ or\ basolateral\ Panx1\ localization\ (\%) = \frac{apical\ or\ basolateral\ FI}{total\ FI} \times 100$$

Data are reported as means ± standard error of the mean (SEM) unless otherwise indicated. All statistical analyses were performed using GraphPad Prism 6.0 software

(GraphPad Software, Inc.). All Student's *t*-tests performed were two-tailed. Statistical significance was noted when $P < 0.05$.

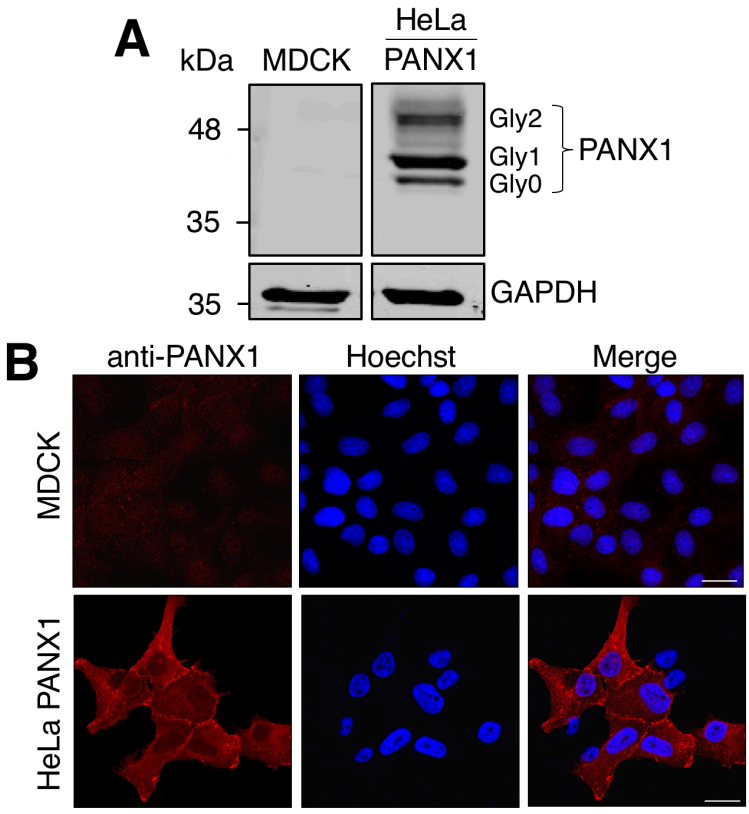
2.3 Results

2.3.1 Panx1 is not detected in MDCK cells

To first characterize the endogenous expression level of Panx1 in non-polarized MDCK cells, cells were lysed and subjected to immunoblotting using a polyclonal antibody directed against the C-terminus of PANX1 (**Fig. 2.1A**). The typical three band pattern representing the glycosylation status of Panx1 (Boassa et al., 2007; Penuela et al., 2007) was not detected in MDCK cells. As a positive control, Panx1 resolved as multiple glycosylated species in the 41 – 48 kDa range in HeLa cells engineered to ectopically express PANX1 (Boassa et al., 2007). The degree of cross-reactivity of the anti-PANX1 antibody with canine Panx1 was not a confounding factor, as the amino acid sequence canine and human Panx1 are identical at the epitope. The lack of endogenous Panx1 expression was further confirmed by immunofluorescent labeling (**Fig. 2.1B**). Cell surface labeling was observed in PANX1-overexpressing HeLa cells, but not in MDCK cells.

2.3.2 MoxGFP-tagged Panx1 localizes predominantly to the apical cell surface of polarized MDCK cells

To investigate the distribution of Panx1 in polarized and non-polarized cells, moxGFP-tagged Panx1 was first stably expressed in non-polar MDCK and BICR-M1R$_k$ cells, which have previously been used to examine Panx1 trafficking (Bhalla-Gehi et al., 2010). In both cell types, Panx1 was detected throughout the plasma membrane, indicating that the fluorescent protein tagged Panx1 was trafficked effectively to the cell surface (**Fig. 2.2A**). In BICR-M1R$_k$ cells, Panx1-moxGFP accumulated at the distal ends of cell surface protrusions that are indicative of invadopodia (**Fig. 2.2A**, bottom row). MDCK cells stably expressing Panx1-moxGFP were subsequently grown on filter supports (in which cells polarized after reaching confluence) and monitored for the establishment of transepithelial electrical resistance (TEER) as a measure of cell polarity. TEER


A
kDa
MDCK
HeLa
PANX1
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35
35
Gly2
Gly1
Gly0
PANX1
GAPDH
B
anti-PANX1
Hoechst
Merge
MDCK
HeLa PANX1

Figure 2.1

Figure 2.1. **Panx1 is not detected in MDCK cells**. (A) MDCK cell lysates were immunoblotted for Panx1 using an anti-PANX1 antibody. HeLa cells ectopically expressing PANX1 were used as a positive control. GAPDH served as a protein loading control. (B) Immunolabeling of Panx1 in MDCK cells (top row) and PANX1-transfected HeLa cells confirmed the absence of endogenous Panx1 in MDCK cells. Scale bars, 20 μm.

measurements revealed that the cells polarized within 4-5 days (**Fig. 2.2B**). Once polarized, cells were fixed and immunolabeled for cortical F-actin, which typically decorates the cell periphery, and occludin, a transmembrane protein found in tight junctions (**Fig. 2.2C**). Analysis of the polarized monolayer in the Z-axis revealed that Panx1 localized predominantly to the apical surface relative to the occludin staining. Analysis of the fluorescence intensity denoting Panx1 and F-actin along a line in the apical-basolateral plane confirmed that the maximum Panx1 signal was visualized with that of F-actin at the apical cell surface (**Fig. 2.2C**). To more clearly ascertain the extent to which Panx1 localized to the apical versus the basolateral domain, cells in Transwells were additionally stained for β-catenin, a marker for the basolateral cell surface (Rice et al., 2015), and the relative fluorescence intensity denoting Panx1 in each membrane domain was quantified (**Fig. 2.2E, F**). Approximately 75% of total Panx1 at the cell surface was detected at the apical domain, while the remaining 25% was localized to the basolateral domain (**Fig. 2.2G**).

In order to further assess the localization of Panx1 in polarized epithelial cells in 3 dimensions, MDCK cells stably expressing Panx1-moxGFP were cultured in Matrigel for 1 week (**Fig. 2.3A**). MDCK cells formed spherical cyst-like structures where occludin was localized to tight junctions at the apical/basolateral barrier (**Fig. 2.3B, arrows**). Similar to cells grown in polarized monolayers, Panx1 was detected largely at the apical surface of the cells (**Fig. 2.3B,** dashed lines), although still present to a much lesser degree at the basolateral membrane domain (**Fig. 2.3B,** arrowheads). This distribution pattern of Panx1 was visible throughout the depth of the spheroid; however, cells trapped within the lumen of the spheroid appeared to have widely distributed Panx1 as it was unclear whether these cells were polarized (**Fig. 2.3C**). Overall, these data indicate that Panx1 localizes preferentially to the apical membrane domain of polarized MDCK cells.

2.3.3 Generation and characterization of moxGFP-tagged Panx1 mutants

Apical domain sorting motifs are less well-defined than basolateral-specific sorting signals; therefore, we focused our attention on determining whether basolateral sorting motifs were encoded within the Panx1 polypeptide that accounted for the 25% of Panx1 protein that was found in the basolateral domain. An analysis of the Panx1 polypeptide

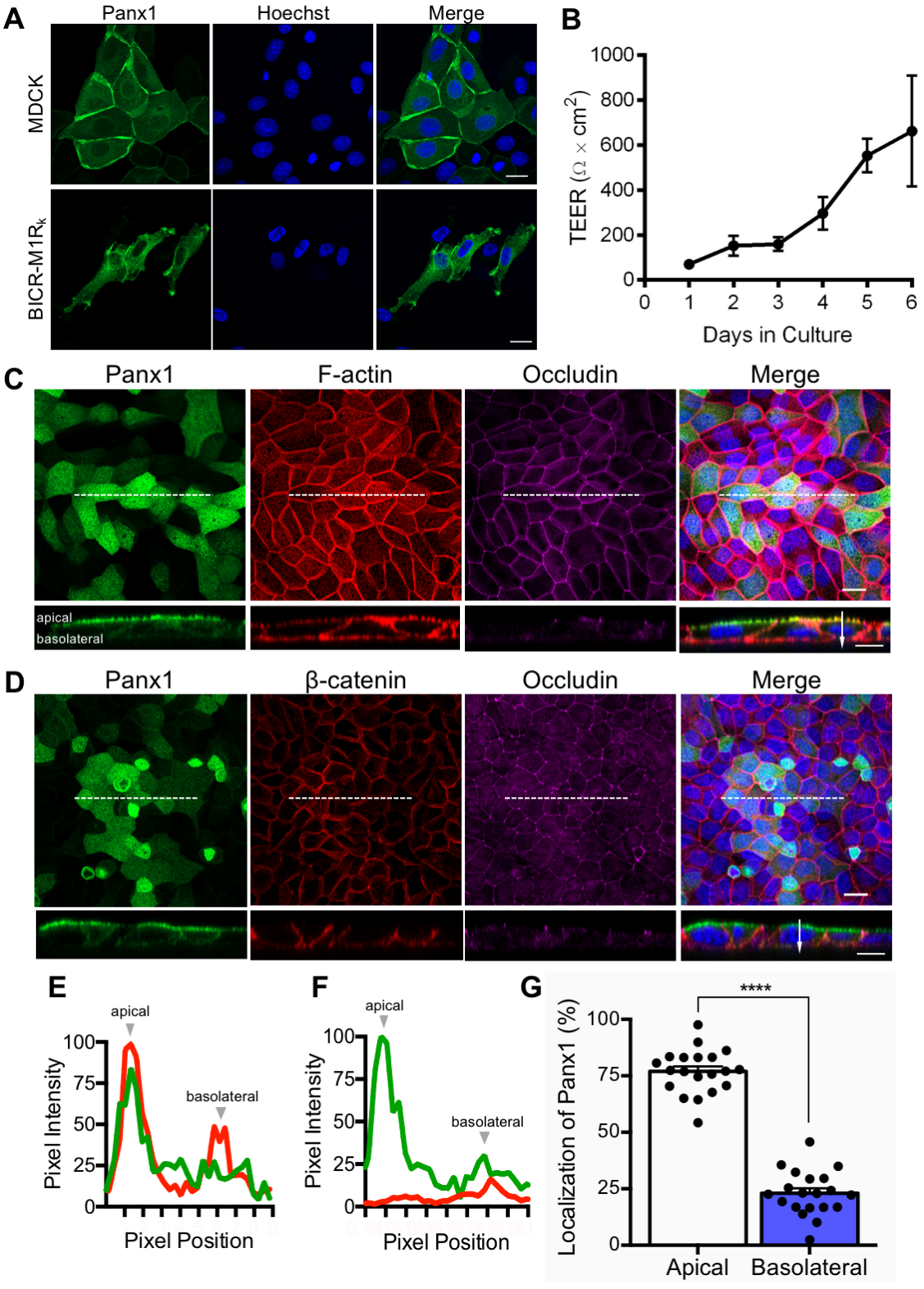
A
Panx1
Hoechst
Merge
MDCK
BICR-M1R$_k$
B
TEER ($\Omega \times cm^2$)
Days in Culture
C
Panx1
F-actin
Occludin
Merge
apical
basolateral
D
Panx1
β-catenin
Occludin
Merge
E
apical
basolateral
Pixel Intensity
Pixel Position
F
apical
basolateral
Pixel Intensity
Pixel Position
G
****
Localization of Panx1 (%)
Apical
Basolateral

Figure 2.2

Figure 2.2. **Panx1 localizes preferentially to the apical surface of polarized MDCK cells**. (A) MoxGFP-tagged Panx1 was efficiently delivered to the plasma membrane of non-polarized MDCK and BICR-M1R$_k$ cells. (B) MDCK cells stably expressing Panx1-moxGFP were cultured in Transwells and TEER across the monolayer was measured daily until the cells developed resistance indicative of a polarized cell monolayer. (C, D) Representative orthogonal projections of MDCK cells after polarization. F-actin (C) and β-catenin (D) were used as surrogate markers of the cell periphery and the basolateral domain, respectively. Occludin was labeled to delineate and denote the position of the tight junctions. Bottom rows represent xz projections of the cells in the apical-basolateral plane along the white dashed line. (E, F) Pixel intensities of Panx1 (green), and either F-actin (E) or β-catenin (F) (red) along the plane of the white arrow in the bottom rows of panels (C) and (D). (G) Quantification of the proportion of Panx1 signal in the apical and basolateral membrane domains. While still detected basolaterally, Panx1 localized largely to the apical surface of MDCK cells. ****P<0.0001, paired t-test. Scale bars, 20 μm.

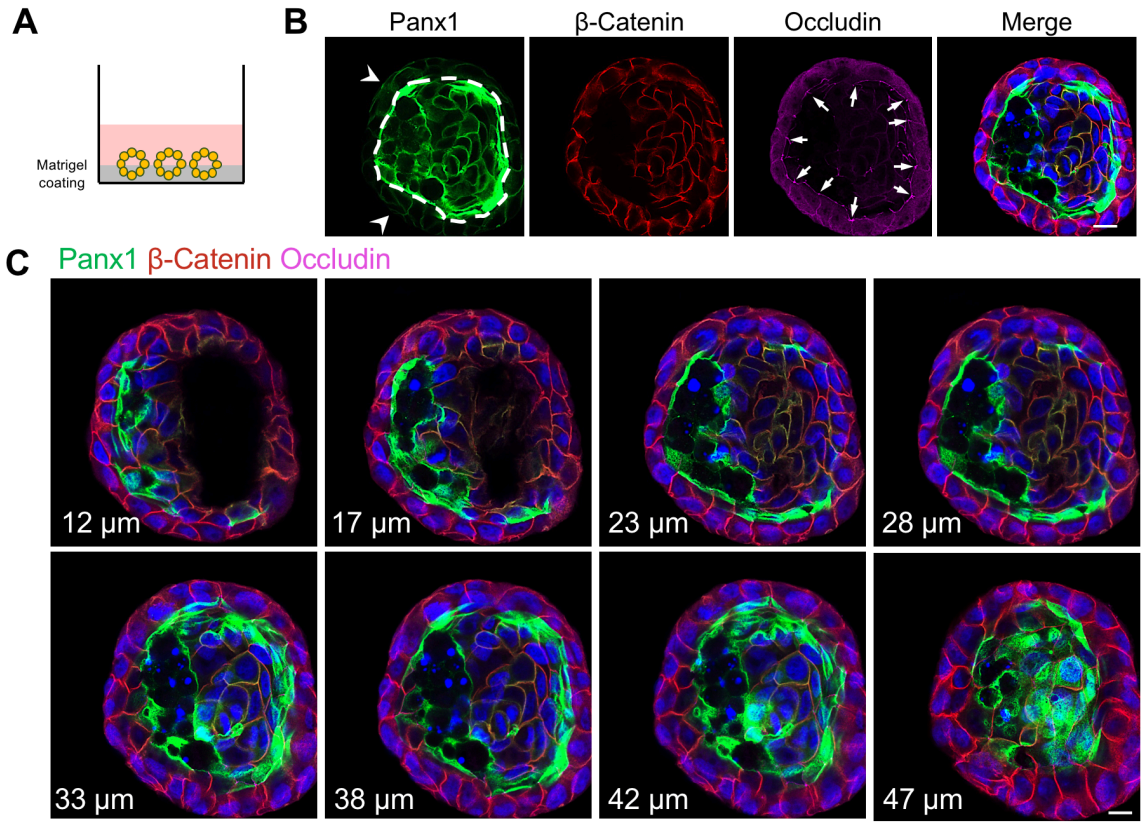
A
Matrigel coating
B
Panx1
β-Catenin
Occludin
Merge
C
Panx1 β-Catenin Occludin
12 µm
17 µm
23 µm
28 µm
33 µm
38 µm
42 µm
47 µm

Figure 2.3

Figure 2.3. Panx1 is apically distributed in spheroids of polarized MDCK cells. (A) Schematic diagram of experimental setup. MDCK cells stably expressing Panx1-moxGFP were seeded as single cells onto Matrigel-coated dishes and grown for 7 days before forming a spheroid, in which a single layer of epithelial cells enclosed a lumen with a few cells trapped within. (B) Representative orthogonal projection of z-stacks through an MDCK cell spheroid. Dashed white lines denote the edge of the spheroid lumen. β-catenin was employed as a basolateral cell surface marker. Arrowheads point to low levels of Panx1 found within the basolateral domain while the bulk of Panx1 was found on luminal side of the spheroid denoted by the position of the tight junction protein, occludin (white arrows). (C) Panel of optical images denoting the spheroid as various z dimensions reveals the apical distribution of Panx1. Scale bars, 20 μm.

sequence revealed a dileucine motif and a tyrosine-containing motif within the C-terminal domain of Panx1, two well-characterized sorting signals found in other basolaterally- destined proteins (Stoops & Caplan, 2014). To eliminate the possibility that other sorting motifs might exist in the C-terminal domain, we decided to truncate Panx1 at residue 379, which is the site of caspase 3-mediated cleavage in cells undergoing apoptosis (Chekeni et al., 2010; Sandilos et al., 2012). Thus, one C-terminal truncation mutant (Δ379) and two point mutants (Y308F and LL365/6AA) were generated to assess the role of the C-terminal domain and putative sorting motifs on Panx1 trafficking and final domain residence in non-polar and polarized cells (**Fig. 2.4A**). First, each mutant was stably expressed in non-polarized MDCK and BICR-M1R_k cells to determine whether any of the mutants would exhibit altered trafficking or whether expression of the mutants would overtly affect the cellular phenotype (**Fig. 2.4B, C**). In the case of MDCK cells, the cells were immunolabeled for calnexin, an ER resident molecular chaperone, to assess whether the Panx1 mutants were retained with the ER. (**Fig. 2.4B**). All three mutants exhibited robust cell surface distribution in both cell lines, although some intracellular Panx1 was also evident. Interestingly, stable expression of Y308F and Δ379 in MDCK cells changed the epithelial-like phenotype of the cells as the cells exhibited an elongated fibroblast-like morphology. However, this phenotypic change was not observed for cells expressing the LL365/6AA mutant (**Fig. 2.4B**).

2.3.4 Expression of the Y308F Panx1 mutant alters the epithelial phenotype of MDCK cells and prevents their polarization

Y308 was recently identified as a Panx1 Src kinase phosphorylation site but could putatively also have a dual function as part of a tyrosine-based basolateral targeting motif (Stoops & Caplan, 2014; Weilinger et al., 2016). Due to the morphological change observed in MDCK cells upon prolonged expression of Y308F, we first assessed whether the behavior of the cells had been altered as well. Non-polarized wild type and Y308F Panx1 expressing cells were labeled for F-actin and β-catenin (**Fig. 2.5A, B**). Interestingly, in Y308F expressing cells, F-actin redistributed from the cell periphery into stress fibres, suggesting that these cells had acquired a more migratory phenotype (**Fig.**

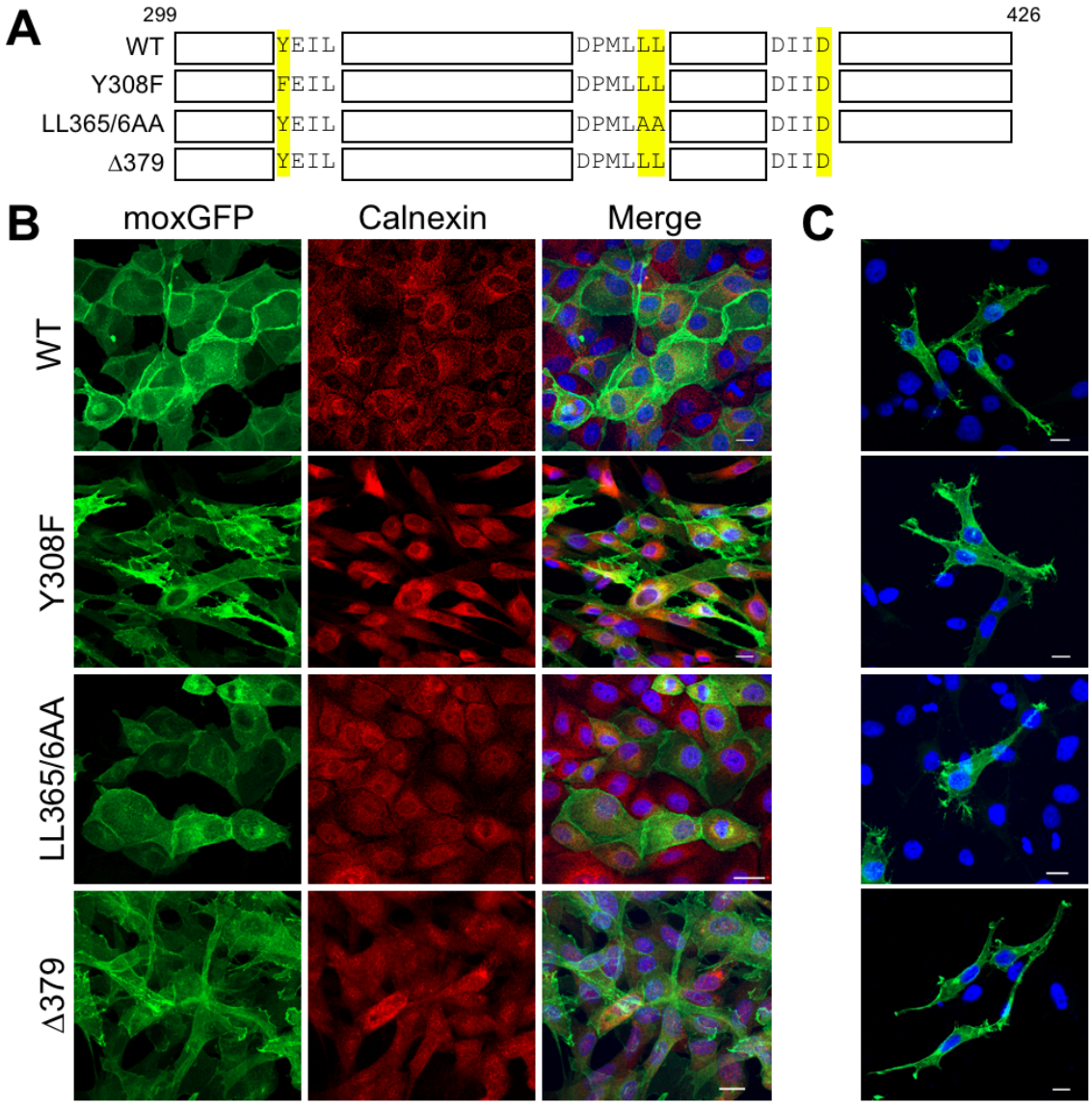
A
299
426
WT
YEIL
DPMLLL
DIID
Y308F
FEIL
DPMLLL
DIID
LL365/6AA
YEIL
DPMLAA
DIID
Δ379
YEIL
DPMLLL
DIID
B
moxGFP
Calnexin
Merge
C
WT
Y308F
LL365/6AA
Δ379

Figure 2.4

Figure 2.4. Characterization of moxGFP-tagged Panx1 mutants in non-polarized cells. (A) Mutations were engineered within the C-terminal domain of Panx1. All Panx1 mutants were tagged with moxGFP at the carboxy terminus. Amino acid residues shown represent putative basolateral targeting motifs. Highlighted residues represent sites of mutation. The extent of the C-terminal domain is denoted by the amino acid residue numbers shown. (B) Non-polarized MDCK cells engineered to stably express each mutant were labeled for calnexin, a resident chaperone protein of the endoplasmic reticulum. All mutants effectively trafficked to the cell surface. MDCK cells expressing the Y308F and Δ379 Panx1 mutants exhibited a switch from an epithelial-like to a fibroblast-like morphology. (C) Panx1 mutants were additionally expressed in nonpolar BICR-M1R$_k$ cells. Note that all mutants were found at the cell surface, including the invadopodia-like structures, and there were no observed changes in cell shapes. Scale bars, 20 μm.

2.5A). In addition, β-catenin relocalized from areas of cell-cell contact into punctate structures throughout the cytoplasm, indicative of a potential loss of its role in cell adhesion (**Fig. 2.5B**). Consistent with MDCK cells losing their epithelial-like phenotype, subsequent culturing of Y308F expressing cells in Transwells revealed that they were unable to polarize as revealed by the inability to establish sufficient TEER, or express detectable levels of occludin (**Fig. 2.5C–E**). Similar to Y308F-expressing MDCK cells grown in non-polarizing conditions, actin stress fibres and β-catenin were detected throughout the cytoplasm of each mutant-expressing cell. To investigate whether the cells had undergone epithelial-to-mesenchymal transition (EMT), wild type Panx1 and Y308F-expressing cells were immunolabeled for E-cadherin, a marker of epithelial cells (Zeisberg & Neilson, 2009) (**Fig. 2.6A**). In wild type Panx1-expressing cells, E-cadherin was localized to the plasma membrane, consistent with its role in adherens junctions but in Y308F expressing cells, E-cadherin was more readily found redistributed with intracellular locations. As might be expected if Y308F expressing cells had acquired a more mesenchymal phenotype, they were found to be positive for N-cadherin (Zeisberg & Neilson, 2009) (**Fig. 2.6B**).

2.3.5 The C-terminal dileucine-based motif does not participate in targeting Panx1 to the basolateral domain of polarized MDCK cells

A LL365/6AA Panx1 mutant was stably expressed in MDCK cells and cultured on membrane filter inserts. Evaluation of TEER in LL365/6AA-expressing MDCK cells in Transwells revealed that the cells retained the ability to polarize *in vitro* (**Fig. 2.7A**). Similar to wild type Panx1, dileucine-mutated Panx1 exhibited a predominantly apical cell surface distribution in these polarized cells (**Fig. 2.7B, C**). Quantitative analysis of the distribution of the LL365/6AA mutant in relation to that of F-actin and β-catenin in the z-axis revealed that 75% of Panx1 mutant localized to the apical domain while 25% continued to be found in the basolateral domain, similar to that found for wild type Panx1 (**Fig. 2.7D, E**). Collectively, these data demonstrate that mutating the C-terminal dileucine-based motif of Panx1 does not affect the targeting of Panx1 to the basolateral surface of polarized MDCK cells.

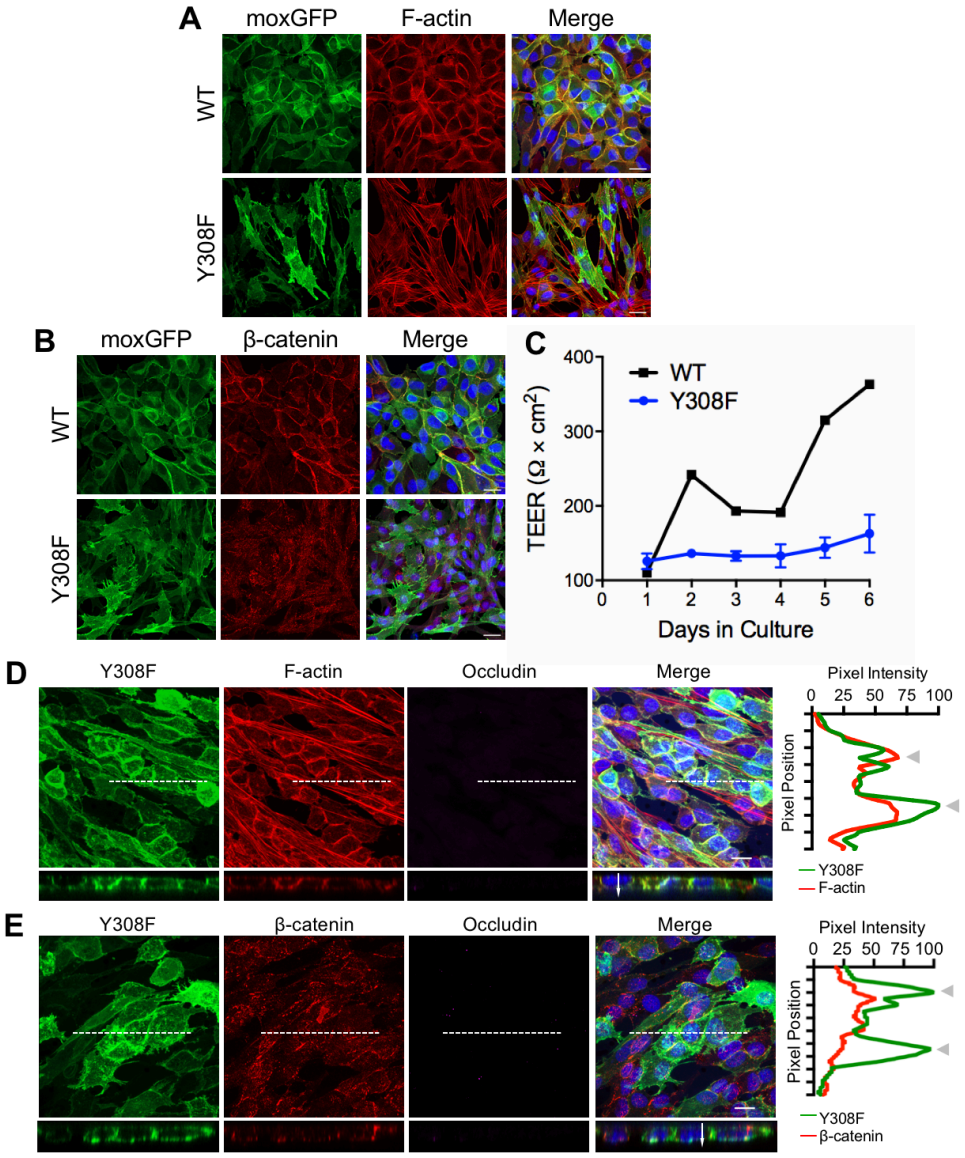
A
moxGFP
F-actin
Merge
WT
Y308F
B
moxGFP
β-catenin
Merge
WT
Y308F
C
WT
Y308F
TEER (Ω × cm²)
400
300
200
100
0
1
2
3
4
5
6
Days in Culture
D
Y308F
F-actin
Occludin
Merge
Pixel Intensity
0 25 50 75 100
Pixel Position
Y308F
F-actin
E
Y308F
β-catenin
Occludin
Merge
Pixel Intensity
0 25 50 75 100
Pixel Position
Y308F
β-catenin

Figure 2.5

Figure 2.5. Expression of the Y308F Panx1 mutant affects the epithelial phenotype of MDCK cells and inhibits cell polarization. (A, B) MDCK cells stably expressing wild type and Y308F Panx1-moxGFP were cultured as non-polarized cells and stained for F-actin (A) and β-catenin (B). Redistribution of F-actin into stress fibres and β-catenin into intracellular sites were observed upon the expression of Y308F. (C) Y308F cells cultured in Transwells displayed no changes in TEER readings over 6 days, indicating that the cells were unable to polarize. One trial of wild type (WT) Panx1-expressing cells were included in parallel and shown to polarize after 5 days of culturing. (D, E) Transwell cultures of Y308F cells were labeled for F-actin (D), β-catenin (E) and occludin. Occludin was not detectable, confirming the lack of epithelial polarity in the cells. Actin stress fibres were visible throughout each cell. β-catenin was distributed intracellularly as punctate-like structures. Top rows (D, E), representative orthogonal projections of cells in Transwells. Bottom rows (D, E), projection of cells along the z-axis in the plane of the white dashed line. Y308F was detected throughout the plasma membrane of each cell. Right panels (D, E), pixel intensity plots of Y308F, F-actin and β-catenin along the line indicated by the white arrow in xz profiles. Grey arrowheads (D, E, intensity plots) indicate areas along the plot corresponding to the plasma membrane. Scale bars, 20 μm.

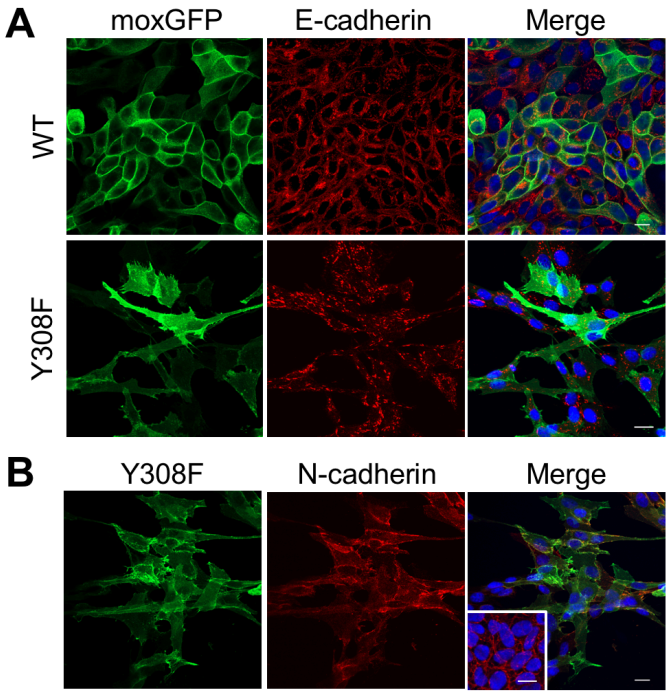

Figure 2.6

Figure 2.6. MDCK cells stably expressing Y308F Panx1 exhibited changes indicative of EMT. Cells stably expressing wildtype and Y308F Panx1-moxGFP were cultured on coverslips and immunolabeled for (A) E-cadherin. E-cadherin was localized to the cell surface in wild type (WT) expressing cells but localized to intracellular sites in cells expressing the Y308F mutant. (B) Y308F mutant expressing cells stained positively for the mesenchymal cell marker N-cadherin. Inset represents AD293 cells immunostained for N-cadherin as a positive control. Scale bars, 20 μm.

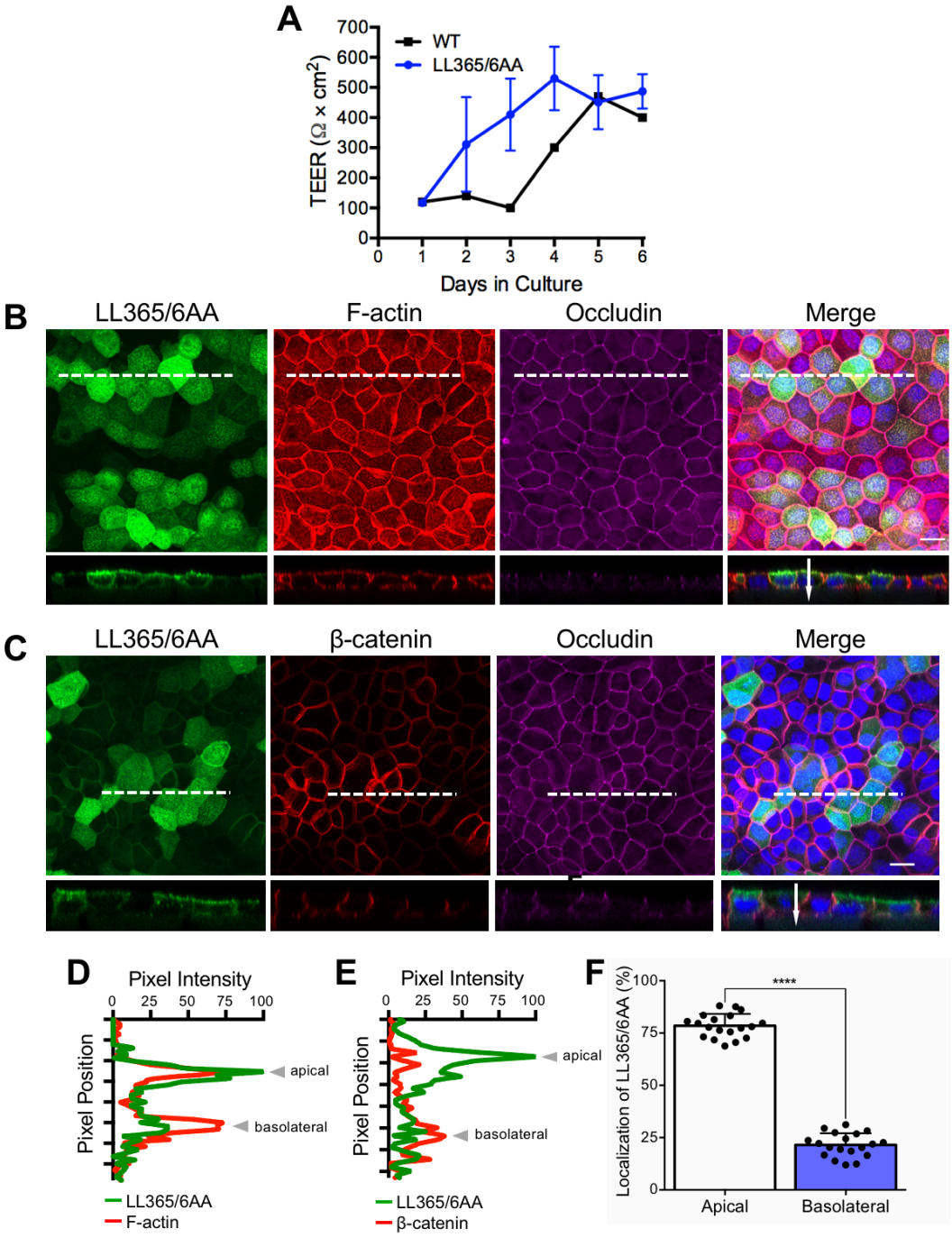
A
WT
LL365/6AA
TEER (Ω x cm²)
Days in Culture
B
LL365/6AA
F-actin
Occludin
Merge
C
LL365/6AA
β-catenin
Occludin
Merge
D
Pixel Intensity
Pixel Position
apical
basolateral
LL365/6AA
F-actin
E
Pixel Intensity
Pixel Position
apical
basolateral
LL365/6AA
β-catenin
F
Localization of LL365/6AA (%)
****
Apical
Basolateral

Figure 2.7

Figure 2.7. Mutation of a C-terminal dileucine-based motif does not alter the distribution of moxGFP-tagged Panx1 in polarized MDCK cells. (A) MDCK cells stably expressing a moxGFP-tagged LL365/6AA Panx1 mutant were cultured in Transwells and subjected to daily TEER measurements until they exhibited electrical resistance consistent with reaching a polarized state. One trial of wild type (WT) cells were included in parallel and shown to polarize after 4-5 days of culturing. (B, C) Representative orthogonal projections of polarized cells after labeling for F-actin (to denote the cell periphery) and β-catenin (to demarcate the basolateral domain). Occludin was also labeled to denote the position of the tight junctions. The xz images represent projections in the apical-basolateral plane along the white dashed line. (D, E) Pixel intensity plots of the LL365/6AA mutant together with either F-actin (D) or β-catenin (E) along the plane denoted by the white arrow in the bottom rows of panels B and C. (F) The proportion of the LL365/6AA mutant in the apical and basolateral domains were quantified. Approximately 25% of the LL365/6AA mutant was detected in the basolateral domain. ****$P<0.0001$, paired *t*-test. Scale bars, 20 μm.

2.3.6 Expression of C-terminally truncated Panx1 inhibits MDCK cell polarization

The C-terminal domain of Panx1 has been previously demonstrated to interact directly with F-actin (Bhalla-Gehi et al., 2010). In addition, this region of Panx1 also contains a caspase cleavage at amino acid 379, which occurs during cellular apoptosis (Chekeni et al., 2010; Sandilos et al., 2012). Previous studies have reported that expression of this cleaved form leads to cell death (Chekeni et al., 2010; Engelhardt et al., 2015). We were encouraged by the fact that the expression of the Δ379 mutant did not lead to MDCK cell death, thus enabling us to assess whether the mutant would affect cell polarization. After generation of a stable cell line of Δ379-expressing MDCK cells, we first examined whether the morphological changes observed in these cells were accompanied with a redistribution of F-actin redistribution. F-actin appeared more dispersed and less peripheral when compared with cells expressing wild type Panx1 (**Fig. 2.8A**). In addition, β-catenin relocalized from sites of cell-to-cell apposition in mutant-expressing cells to more intracellular locations consistent with a change in cell-cell adhesion properties (**Fig. 2.8B**).

To assess the effect of expressing the Δ379 mutant on the ability of MDCK cells to polarize, MDCK cells stably expressing Δ379 were plated on filter inserts and TEER was monitored daily. TEER did not change over 6 days suggesting that mutant expressing MDCK cells did not polarize (**Fig. 2.9A**). Subsequent labeling of these Panx1 mutant expressing MDCK cells for F-actin and β-catenin revealed the same distribution patterns as observed when these cells were grown on glass coverslips (**Fig. 2.9B, C**). Intriguingly, since these cells failed to polarize they also failed to upregulate the expression of the tight junction protein, occludin. (**Fig. 2.9B, C**). Overall, these results indicate that prolonged expression of Δ379-truncated Panx1 induces reorganization of the actin cytoskeleton and ablation of the ability of MDCK cells to polarize *in vitro*.

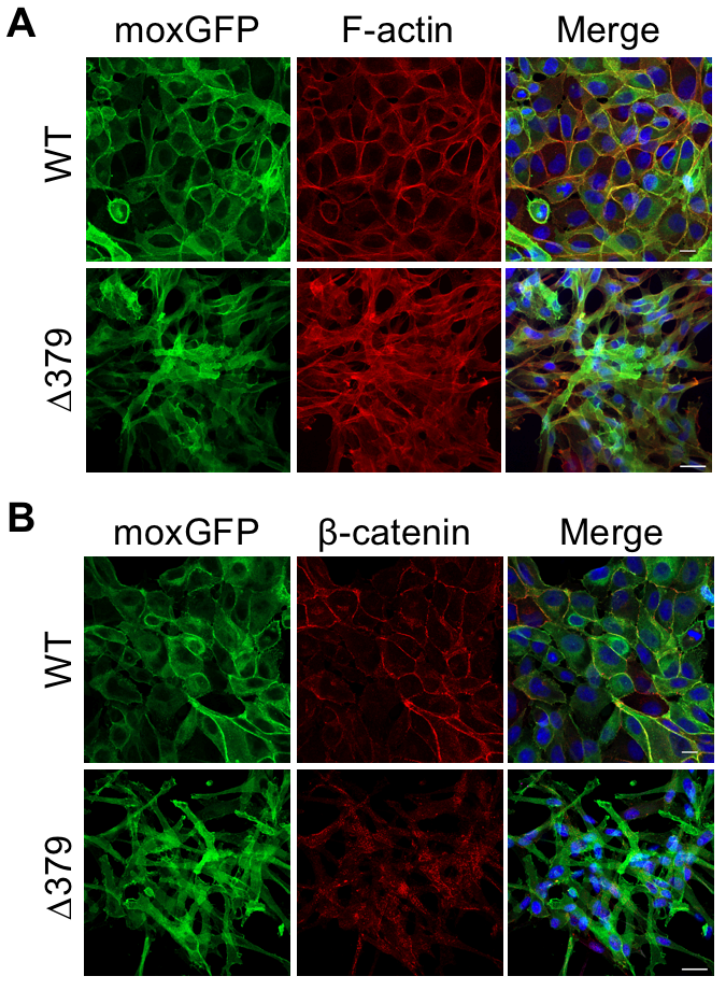
A
moxGFP
F-actin
Merge
WT
Δ379
B
moxGFP
β-catenin
Merge
WT
Δ379

Figure 2.8

Figure 2.8. MDCK stably expressing Δ379-truncated Panx1 exhibit changes in the distribution of F-actin and β-catenin. MDCK cells expressing wildtype (WT) and truncated moxGFP-tagged Panx1 were cultured on coverslips and labeled for (A) F-actin and (B) β-catenin. Compared to wildtype, prolonged expression of truncated Panx1 resulted in the redistribution of both F-actin and β-catenin. Scale bars, 20 μm.

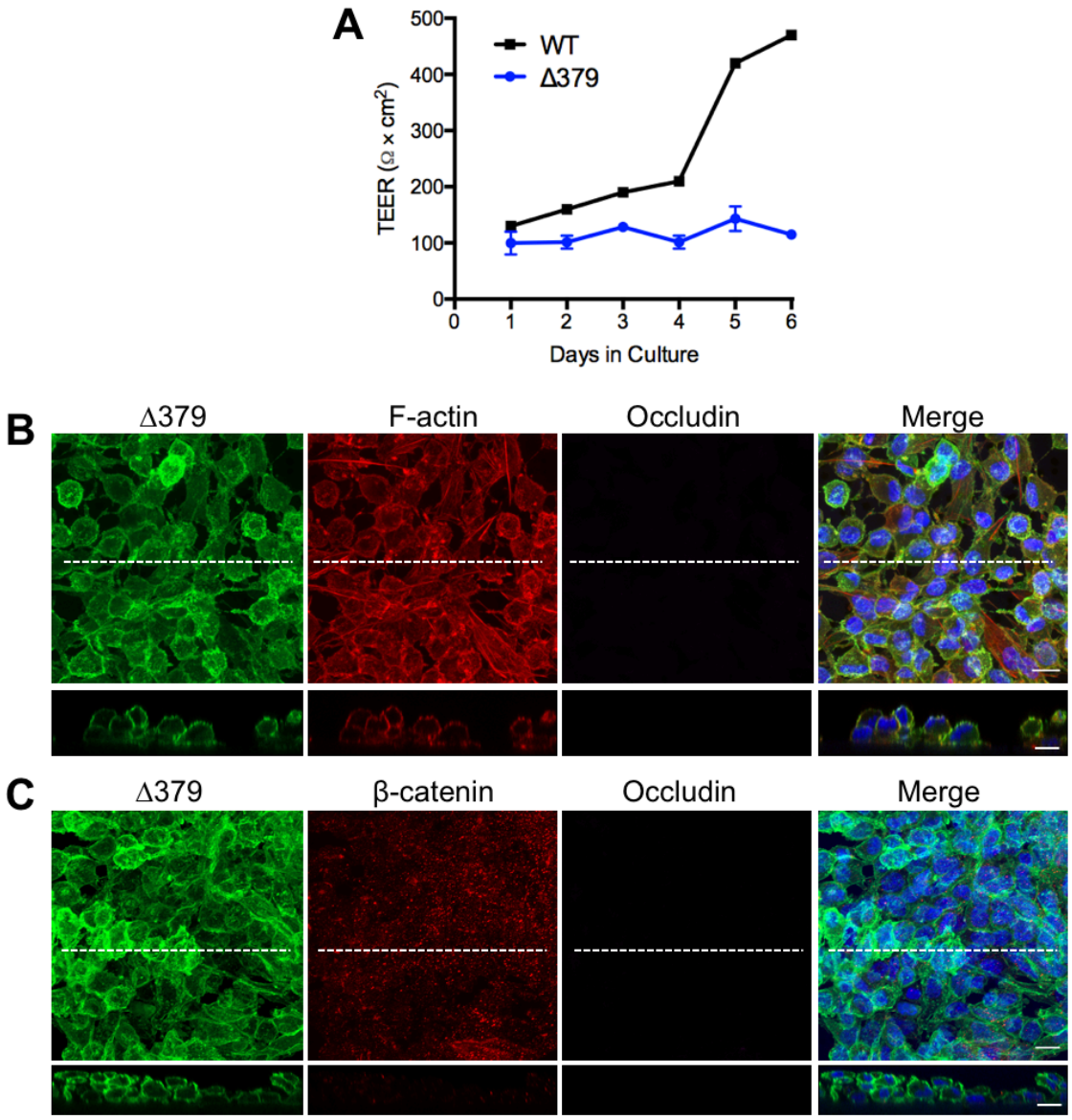
A
500
400
300
200
100
0
TEER (Ω x cm²)
WT
Δ379
0
1
2
3
4
5
6
Days in Culture
B
Δ379
F-actin
Occludin
Merge
C
Δ379
β-catenin
Occludin
Merge

Figure 2.9

Figure 2.9. Expression of Δ379 truncated Panx1 disrupts the ability of MDCK cells to polarize. (A) TEER measurements of Δ379 mutant expressing cells cultured in Transwells reveal that they were unable to polarize. One trial of wild type (WT) Panx1-expressing cells were included in parallel and shown to polarize after 4-5 days of culturing. (B, C) Δ379 mutant expressing cells in Transwells were stained for occludin and either F-actin (B) or β-catenin (C). Bottom rows (B, C) represent cell projections along the z-axis in the plane of the white dashed line. Cells were devoid of occludin expression consistent with the inability of the mutant expressing cells to polarize. Scale bar, 20 μm.

2.4 Discussion

Panx1 is expressed in most mammalian tissues and cell types and is abundantly found in epithelial-rich organs such as the kidneys, intestines, liver, skin, testes and the bladder (Le Vasseur et al., 2014; Negoro et al., 2013; Penuela et al., 2007; Penuela et al., 2013; Turmel et al., 2011). Most studies investigating Panx1/PANX1 gene expression in human tissues rely on screening for mRNA, which does not inform on protein localization at the cellular level or distinguish between polarized and non-polarized cell types within the tissue. In addition, reliable and readily available Panx1/PANX1-specific antibodies have been difficult to obtain in the past; thus, the precise localization of Panx1 in polarized epithelial cells remains poorly understood. Here, we demonstrate for the first time that Panx1 acquires a predominantly apical distribution in polarized MDCK cells in an *in vitro* model of epithelia, and that mutations in the Panx1 polypeptide can affect the epithelial cell phenotype, including their ability to polarize.

In the present study, we first examined the localization of Panx1 in non-polar MDCK and BICR-M1R$_k$ cells. As might be expected, Panx1 was localized throughout the entire plasma membrane of both cell types, as well as in intracellular compartments. However, in BICR-M1R$_k$ cells, Panx1 was enriched specifically at the distal finger-like protrusions of the plasma membrane indicative of invadopodia, suggesting that Panx1 channels may be functionally involved in processes and structures that drive cell migration. Previously, Panx1 activity has been linked to cell migration in keratinocytes and T lymphocytes, where reduced Panx1 levels was associated with delayed wound closure and reduced cell motility upon lymphocyte activation (Penuela et al., 2014; Velasquez et al., 2016). Furthermore, it has been reported that ATP release from Panx1 channels drives reorganization of the actin cytoskeleton, and that this remodeling is required for the homing of dendritic cells to lymph nodes (Saéz et al., 2017). Thus, the involvement of Panx1 in cell migration is likely a consequence of both channel function and changes in cytoskeletal arrangement.

Upon polarization of MDCK cells, Panx1 was predominantly found in the apical domain, with only 25% being detected at the basolateral domain. The reason why this population of Panx1 is continually found at the basolateral domain is not clear, but it is possible that

Panx1 molecules targeted to this domain could still be retrieved and sent to the apical domain through transcytosis (Fung et al., 2017; Mostov et al., 2000; Odorizzi, Pearse, Domingo, Trowbridge, & Hopkins, 1996). The predominance of Panx1 at the apical membrane domain supports the view that Panx1 most likely functions as single membrane channel protein, as opposed to an early report that it might form intercellular gap junction channels similar to connexins (Bruzzone et al., 2003). Thus, our data support the notion that the overall cellular function of pannexins is distinct from that of the related connexin family of gap junction proteins.

2.4.1 Effects of expressing Panx1 mutants on the epithelial cell phenotype

The fact that Panx1 is largely found at the apical domain, with approximately 25% being observed in the basolateral domain, raises questions as to whether molecular sorting motifs are encoded within the Panx1 polypeptide. Apical sorting signals of membrane proteins are highly variable in cells, and can reside in any topological domain of integral membrane proteins (Stoops & Caplan, 2014; Weisz & Rodriguez-Boulan, 2009). The composition of such motifs also varies; depending on the protein and cell type, apical sorting motifs can take on the form of amino acid sequences, of which consensus sequences are poorly defined, glycosylphosphatidylinositol (GPI)-anchors and glycosylation moieties (Weisz & Rodriguez-Boulan, 2009). Others have previously shown that glycosylation-deficient Panx1 mutants are largely unable to traffic to the plasma membrane of several non-polarized cell types, suggesting that these carbohydrate moieties may not specifically direct the sorting of Panx1 to either the apical or basolateral domain (Boassa et al., 2008; Penuela et al., 2009). Moreover, there is no evidence that Panx1 undergoes post-translational GPI-anchoring, but this has yet to be fully investigated. Here, we chose to examine the importance of putative sorting motifs that may direct Panx1 to the basolateral domain which appears to occur ~25% of the time in MDCK cells (Stoops & Caplan, 2014). Since the C-terminal tail of Panx1, as a whole, is indispensable for cell surface trafficking (Gehi et al., 2011), we selectively mutated two motifs that correspond to signature basolateral-specific targeting motifs (Stoops & Caplan, 2014; Weisz & Rodriguez-Boulan, 2009).

We first found that stable expression of the Y308F mutant of Panx1 was associated with a loss in the ability of the cells to polarize *in vitro* and concomitant acquisition of a fibroblast-like morphology that is indicative of EMT. This mutation simultaneously disrupts a tyrosine-based YxxØ consensus motif in the C-terminal domain (where Ø represents a bulky hydrophobic residue), which targets select proteins to the basolateral membrane domain in polarized MDCK cells, and additionally ablates the hydroxyl group present at Y308 making it unacceptable for phosphorylation (Stoops & Caplan, 2014; Zhang et al., 2015). Tyrosine residues are crucial to the binding of adaptor proteins for cargo proteins in endocytosis and in vesicular transport, and mutation of such residues, into even the structurally similar amino acid phenylalanine, disrupts this interaction (Bonifacino & Dell'Angelica, 1999; Mardones et al., 2013; Ohno et al., 1996). In addition, Y308 was recently identified as a Panx1 phosphorylation site for Src family kinases, and this phosphorylation is required for excitotoxic Panx1 channel opening in N2a cells (Weilinger et al., 2012; Weilinger et al., 2016). While it is not clear if Panx1 phosphorylation plays a role in MDCK cells, increased activity of Src family kinases has been extensively linked to changes in cell adhesion, migration and cytoskeletal restructuring in epithelial cells (Avizienyte & Frame, 2005; Behrens et al., 1993). It was particularly intriguing to see that cells expressing the Y308F mutant additionally expressed the mesenchymal marker N-cadherin, and displayed extensive reorganization of the actin network from cortical bundles to contractile stress fibres, both of which are processes commonly associated with EMT (Wheelock et al., 2008; Haynes et al., 2011). Hence, it was not surprising that these Y308F mutant-expressing MDCK cells consistently failed to polarize *in vitro*. At present, no direct link has been drawn between Panx1 and EMT; however, Panx1 overexpression has been implicated in a number of cancers as a tumor suppressor and is known to play a crucial role in carcinogenesis, likely owing to its involvement in cell death, inflammation and autophagy (Chekeni et al., 2010; Furlow et al., 2015; Jiang & Penuela, 2016; Lai et al., 2007). In these cases, however, it is wild type Panx1 that was associated with these processes, and it remains unclear how the Y308F Panx1 mutant would drive EMT. Our findings in MDCK cells represent the first report of a potential connection between EMT and a Panx1 mutant channel.

2.4.2 The C-terminal dileucine motif of Panx1

Upon mutation of a second putative sorting motif, we found that stable expression of the LL365/6AA Panx1 mutant did not disrupt the ability of MDCK cells to polarize and the cells maintained an epithelial-like phenotype. The LL365/6 region of the Panx1 C-terminal domain conforms to the DxxxLL consensus motif, which represents a well-defined sorting signal for several basolaterally-destined proteins in MDCK cells (Kelly et al., 2008; Miranda et al., 2001; Miyashita & Ozawa, 2007; Stoops & Caplan, 2014). However, to our surprise, we found that the proportion of the LL365/6AA mutant Panx1 population that localizes to the basolateral cell surface of polarized cells was nearly identical to that of wild type Panx1, suggesting that this putative sorting motif plays no role in sorting Panx1 to the basolateral domain.

Since neither a tyrosine-based motif or the dileucine motif appear to solely be accountable for targeting Panx1 to basolateral domains we chose to investigate the effect of expressing a Panx1 mutant in which an entire region of the distal C-terminal domain was removed. We selected to truncate Panx1 at amino acid residue 379 for two reasons: first, truncation of Panx1 at the more upstream position T307 causes retention of the mutant in the endoplasmic reticulum (Gehi et al., 2011); and, secondly, Δ379 represents the residual product of caspase-mediated cleavage of Panx1 during apoptosis (Chekeni et al., 2010; Gehi et al., 2011). Of concern in our design was the prior knowledge that this cleavage event renders the channel constitutively open via removal of the autoinhibitory tail of the C-terminus (Chekeni et al., 2010; Sandilos et al., 2012), thus potentially leaving MDCK cells susceptible to cell death. In Jurkat cells, open Panx1 channels have been shown to mediate the release of ATP, which then acts as a recruitment signal for phagocytes to facilitate clearance of apoptotic cells (Chekeni et al., 2010). Furthermore, transient expression of this truncation mutant has been shown to cause widespread cell death in a wide variety of other cell types (Chekeni et al., 2010; Poon et al., 2014; Engelhardt et al., 2015). However, to our surprise, MDCK cells remained largely viable upon stable expression of Δ379 Panx1. These cells elongated, exhibited altered distribution of β-catenin, and failed to polarize. Thus, in this manner, Δ379 Panx1 mutant expressing cells were reminiscent of Y308F-expressing cells, which overtly displayed

markers of EMT. In addition, previous studies have demonstrated that EMT confers cellular resistance to apoptosis in the context of cancer, raising the possibility that this phenotypic change may have offered some resistance to Δ379-expressing MDCK cells, thus protecting them from cell death (Robson et al., 2006; Savagner, 2010; Valdes et al., 2002). Alternatively, the C-terminal moxGFP tag may have sterically acted to block the Panx1 channel pore, thus inhibiting the uncontrolled loss of small intracellular molecules. Consistent with this notion is the fact that all previous studies that showed that the Δ379-truncated Panx1 mutant induced apoptosis employed untagged Panx1 (Chekeni et al., 2010; Engelhardt et al., 2015; Poon et al., 2014; Qu et al., 2011).

2.4.3 Significance of Panx1 channels at the apical membrane domain

Largely owing to the diversity of human pathologies that involve the dysregulation of Panx1, it is becoming increasingly apparent that Panx1 channel function is crucial to normal cell and tissue homeostasis. In this study, we demonstrated that Panx1 assumes a largely apical membrane distribution profile in polarized MDCK cells; thus, we propose that Panx1-mediated release of ATP in polarized epithelia occurs primarily into the luminal space of epithelial-lined cavities. In this cellular context, ATP release may have several tissue-specific functions *in vivo*. For example, stimulation of apical purinergic receptors in the airway epithelium triggers ciliary beating in order to facilitate movement of mucus (Davis & Lazarowski, 2008). Collectively, our results contribute to the body of understanding surrounding the study of Panx1 in health and disease contexts.

In conclusion, our data sheds light into the trafficking and distribution of Panx1 in polarized epithelial cells. We showed that Panx1 is trafficked with substantial preference to the apical membrane domain of polarized MDCK cells, although a residual population remains localized to the basolateral cell surface. Basolaterally-trafficked Panx1 is sorted independently of the C-terminal dileucine motif. Interestingly, we found that a Y308F Panx1 mutant and, possibly a Δ379-truncated mutant, drive MDCK cells toward EMT, as stable expression of either mutant caused phenotypic shifts and loss of epithelial cell characteristics.

2.5 References

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3 General Discussion

The asymmetric organization of membrane proteins and lipids in polarized epithelial cells enables them to perform specialized functions in each cell surface domain. The apical domain of simple epithelial cells faces toward the luminal space of the tissue and is the site of molecular exchange between the organism and the external environment, whereas the basolateral domain mediates cell-cell adhesion, signaling and contact with the underlying substrata (Rodriguez-Boulan & Nelson, 1989). Owing to the dissimilarities in function between each membrane compartment, epithelial cells must carefully regulate protein trafficking and vesicular transport pathways in order to maintain cell surface polarity. Polarized protein sorting acts as a mechanism by which polarized epithelial cells can additionally regulate the functional role of membrane components by selectively sorting them to the apical and/or basolateral domains depending on cell and tissue requirements. In the present study, we assessed the distribution of Panx1 channels in polarized and non-polarized cells and examined the effects of select mutations of Panx1 on the epithelial cell phenotype *in vitro*. Here, we will highlight the functional significance of our findings on the field of pannexin biology as a whole and discuss possible directions for future research.

3.1 Functional implications of membrane domain-specific Panx1 localization in polarized epithelial cells

Given our observation that Panx1 localizes largely to the apical membrane domain in polarized MDCK cells, we propose that Panx1-mediated cell communication occurs preferentially into the luminal cavity of most epithelial-lined organs. As ATP release is the most well-studied molecule to pass through Panx1 channels, we will focus this discussion on the role of purinergic signaling in apical regions of epithelial tissues. Our findings are in keeping with earlier studies in which ATP release from airway epithelial cells was found to depend heavily on apically-localized Panx1 channels (Ransford et al., 2009; Seminario-Vidal et al., 2011). In this context, ATP activates apical P2Y purinergic receptors in the epithelial cells, which then regulates mucociliary clearance via ciliary movement and secretion of mucin and electrolytes into the airway surface liquid (Davis & Lazarowski, 2008). In the renal epithelium, in which Panx1 protein is concentrated

along the apical brush border, stimulation of P2X and P2Y purinergic receptors by luminal ATP effects widespread changes in tubular permeability throughout the nephron (Bailey et al., 2012; Hanner et al., 2012). Finally, in umbrella cells of the rat urothelium, ATP release by apical Panx1 channels was linked to increased frequency of voiding contractions in the bladder and was proposed to occur via the P2 family of purinergic receptors (Beckel et al., 2015).

In this study, we found that although Panx1 was primarily localized to the apical membrane domain, a small but significant population of Panx1 protein remained detectable at the basolateral cell surface. Little is known about the possible functional roles of ATP release at the basolateral microenvironment of epithelia. Tissue types in which Panx1 is documented to localize to the basolateral cell surface include the colon and the epididymis, where purinergic signaling has been functionally linked to apical NaCl secretion by modulating principal cell function (Cyr, 2011; Le Vasseur et al., 2014; Leipziger et al., 1997; Turmel et al., 2011). Panx1 was detected in the basolateral cell surface in addition to the apical domain in urothelial umbrella cell, thus decorating the entire plasma membrane (Beckel et al., 2015). Intriguingly, only apical ATP release from these cells was sensitive to the administration of the Panx1 channel antagonist Brilliant Blue CFC, while basolateral ATP release was unaffected by intravenous treatment (Beckel et al., 2015; Wang et al., 2013). This raises the very fascinating possibility that epithelial cells can selectively and differentially regulate Panx1 channel activity dependent on the membrane domain in which it resides.

Another explanation for the population of Panx1 being observed at the basolateral cell surface may reflect a temporary residence of Panx1 channels in this domain during its transit to the apical plasma membrane. Many epithelial cells employ transcytosis, a process by which epithelial cells can transport vesicular cargo between apical and basolateral membrane domains. Transcytosis is studied largely in the context of transcellular delivery of secretory proteins (Apodaca et al., 1994; Fung et al., 2017). This pathway is also used by epithelial cells as an “indirect” pathway for select proteins to reach its final destination at the apical cell surface (Weisz & Rodriguez-Boulan, 2009). In this pathway, proteins at the basolateral surface are endocytosed and subsequently

delivered to the apical membrane. In addition, the apical and basolateral endocytic pathways of MDCK cells are known to intermix extensively (Apodaca et al., 1994; Odorizzi et al., 1996; Perez Bay et al., 2016; Wang et al., 2000). Thus, Panx1 may be apically trafficked via this indirect route, thus acquiring transient residency at the basolateral domain prior to final steady state delivery to the apical domain.

Previously, members of our group have demonstrated that Panx1 does not co-immunoprecipitate with AP-2, a central component of clathrin-mediated endocytosis (CME) (Gehi et al., 2011). Dileucine consensus motifs are known to frequently serve as binding sites for AP-2 during the endocytic retrieval of transmembrane proteins from the cell surface (Kelly et al., 2008). Thus, our finding that the mutation of the dileucine motif in Panx1 bears no consequence on its distribution in polarized cells is in keeping with prior reports that Panx1 internalization is independent of CME. Furthermore, we propose that this motif does not function in polarized Panx1 protein trafficking.

3.2 Possible role of mutant Panx1 in EMT

In our study, we were surprised to discover that the expression of the Y308F Panx1 mutant caused MDCK cells to transition from epithelial-like to mesenchymal-like cells, a process akin to EMT. The question arises as to how mutant Panx1 would drive the EMT process. As wild type MDCK cells did not express any endogenous Panx1, we doubt that the lack of ATP-mediated purinergic signaling acts to drive MDCK cells to undergo EMT. Instead, it is possible that Panx1 may have a channel-independent influence on EMT progression that may be rooted in the Panx1 interactome. Although channel-independent functions of Panx1 in the context of cancer are largely under-investigated, the breadth of the Panx1 interactome has grown substantially. Known binding partners of Panx1 include Panx2, Panx3, actin, the actin nucleator Arp2/3, caspase-3, caspase-7 and members of the inflammasome complex (NLRP1, ASC, caspase-1, XIAP, and caspase-11) (Chekeni et al., 2010; Gehi et al., 2011; Penuela et al., 2013; Sandilos et al., 2012; Silverman et al., 2009; Wicki-Stordeur & Swayne, 2013, 2014). Of particular relevance to EMT is the interaction between the Panx1 C-terminal domain and the actin cytoskeletal network, the latter of which mediates cell migration by forming membrane protrusions which ultimately mature into invadopodia (Gehi et al., 2011; Wicki-Stordeur

& Swayne, 2013). We have consistently found that Panx1 concentrates at the edges of distal membrane protrusions in migratory and non-polarized BICR-M1Rk cells, both in previous studies (Bhalla-Gehi et al., 2010) and in this present report, supporting the role of Panx1 in cell migration. Indeed, stimulation of migration in neutrophils has been shown to cause Panx1 to translocate to the leading edge of each cell (Chen et al., 2010). Furthermore, inhibition of Panx1 channels with carbenoxolone or with the blocking peptide ^{10}Panx1 severely impaired neutrophil chemotaxis and host defense to bacterial infection *in vivo* (Chen et al., 2010).

We additionally demonstrated that MDCK cells remain viable upon stable expression of the Δ379 truncation mutant of Panx1. The distal C-terminal tail of Panx1 is proposed to gate the channel by acting as a pore plug in a ball-and-chain model; by removing this portion of Panx1, the channel is reported to be constitutively active (Dourado et al., 2014; Sandilos et al., 2012). The open conformation of this channel is believed to increase membrane permeability, leading to cell death (Chekeni et al., 2010; Qu et al., 2011; Sandilos et al., 2012; Engelhardt et al., 2015). In our study, viable MDCK cells stably expressing the truncation mutant exhibited drastic phenotypic changes. Therefore, we proposed two possible reasons for this observation: (1) that the C-terminal moxGFP tag may have functioned as a substitute for the distal C-terminal tail, such that the resultant Panx1 channels function largely as closed membrane pores; and (2) that the cells had become resistant to Δ379-induced cell death by acquiring an EMT-like phenotype. Our data appear to more accurately conform to the latter, as the cells failed to polarize *in vitro* and exhibited indications of altered cell adhesion characteristics.

3.3 Conclusions and future directions

Taken together, our findings represent the first in-depth investigation of Panx1 in polarized cells that provides insight into the trafficking and distribution of Panx1 in epithelial cells. In particular, we demonstrated that Panx1 is largely distributed to the apical membrane domain in polarized cells, and that this sorting is independent of the canonical dileucine-based basolateral targeting signal. Furthermore, we showed for the first time that alterations in specific motifs of Panx1 can induce strong phenotypic transitions in epithelial cells that are indicative of EMT. Collectively, our data highlight

new functional roles of Panx1 in epithelial cells which have never been previously documented.

A number of major questions persist as implications of our study on Panx1 in polarized epithelial cells. First, it remains to be determined whether the residual proportion of basolaterally-distributed Panx1 exists as a transient population in steady state as a consequence of transcytosis, or whether these basolaterally-sorted channels are functionally and terminally destined to reside at this cell surface compartment. In both cases, the exact motifs involved in this delivery have yet to be elucidated, although it is clear from our data that this sorting does not rely on the dileucine motif. Secondly, the distribution of Panx1 in polarized epithelial cells *in vivo* must be re-assessed in light of improved and more reliable reagents and protocols, as several previous studies relied on reagents of questionable reliability. In addition, further work must be done to confirm that expression of Y308F and Δ379 Panx1 induce EMT by expressing the mutants in epithelial-derived cell lines other than MDCK cells, such as Caco-2 cells. This would also allow investigation into whether Panx1 would be trafficked apically in an epithelial cell line derived from the colon, in which Panx1 is documented to reside basolaterally *in vivo* (Le Vasseur et al., 2014). Additionally, it is necessary to further characterize the mesenchymal phenotype of Y308F- and Δ379-expressing MDCK cells. This can be done by comparing the levels of EMT-associated transcription factors, such as Twist, Zeb, Snail and Slug, in wild type, Y308F and Δ379 Panx1-expressing cells via immunoblot analysis. Functional assays, such as migration and invasion assays, of such cells may be used to assess the phenotypic extent of this mutant Panx1-induced EMT. Furthermore, these studies can be replicated in the Caco-2 cell line to determine whether these Panx1 mutants also drive phenotypic transitions in other epithelial cell types. Finally, we can begin to investigate the mechanism behind this EMT induction, which will likely reveal new channel-independent functions of Panx1 and help establish a new perspective on the distribution and diverse cellular roles of Panx1 in polarized epithelial cells.

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Curriculum Vitae

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Honors and Awards

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